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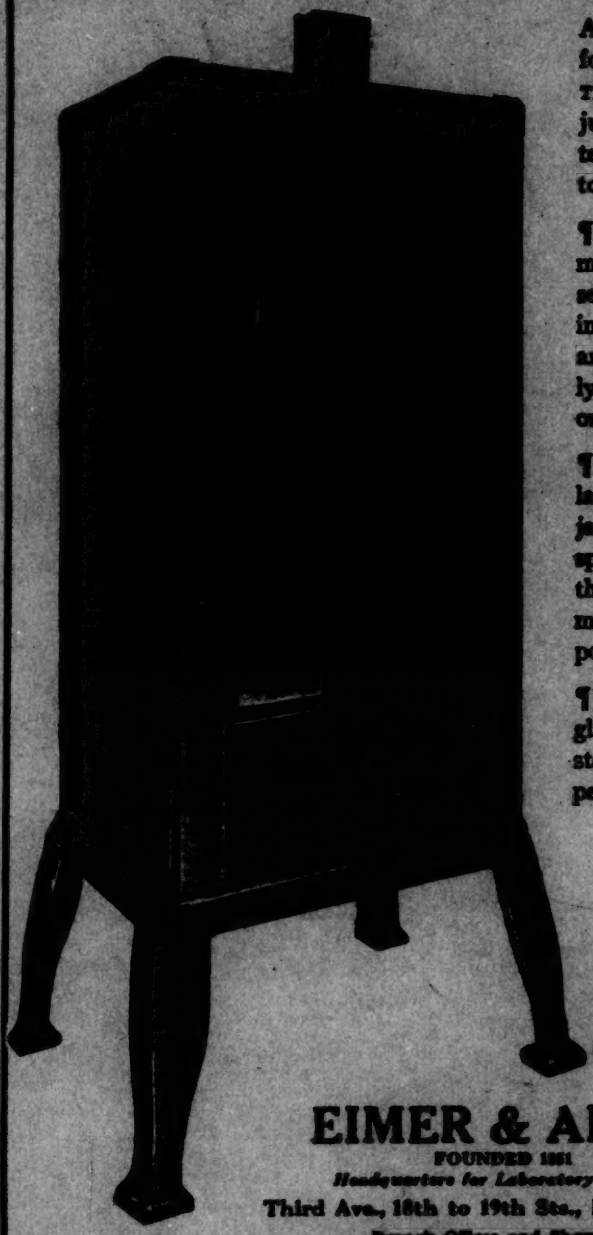

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A MODIFICATION OF THE McLEAN-VAN SLYKE METHOD FOR THE DETERMINATION OF CHLORIDES IN BLOOD.

By G. L. FOSTER.

(*From the Biochemical Laboratory of Harvard Medical School, Boston.*)

(Received for publication, July 19, 1917.)

The most satisfactory method for the determination of chlorides in blood is the one devised by McLean and Van Slyke.[1] This procedure involves removal of the blood proteins by heat, acetic acid, magnesium sulfate, and blood charcoal; precipitation of the chlorides from an aliquot part of the filtrate by means of a dilute standard silver nitrate solution containing nitric acid; removal of the precipitated silver chloride, and titration of the excess silver with dilute standard potassium iodide in the presence of nitrous acid and starch—the free nitric acid being, for the most part, neutralized by the addition of trisodium citrate. The end-point is extremely sensitive and the procedure gives accurate results with very small amounts of chloride.

This method, however, depends in part on the use of Merck's Blood Charcoal Reagent, which at the present time is not to be had. According to McLean and Van Slyke no other charcoal is satisfactory.

We have found that thoroughly reliable results are obtained by applying the original McLean-Van Slyke titration to the filtrates obtained after coagulating the blood proteins with metaphosphoric acid. The additional acidity due to the metaphosphoric acid is not sufficient to interfere with the sensitiveness of the starch-iodine end-point, being only about 2 per cent of that due to the nitric acid.[2] The coagulum formed by the addition of metaphosphoric acid to the diluted blood or plasma is very finely divided, and the diffusion of chlorides to uniform concentration through-

[1] McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, 1915, xxi, 361.

[2] McLean and Van Slyke, *J. Am. Chem. Soc.*, 1915, xxxvii, 1128.

out the mixture is complete in 10 minutes if the flask is shaken occasionally. Our metaphosphoric acid, a J. T. Baker Chemical Company preparation, is free from chlorides.

The method, with the suggested modification, is described in full for the sake of completeness, though the operations after removing the blood proteins are simply those of McLean and Van Slyke, as also are the solutions used in the titration of the protein-free filtrate. We proceed as follows.

Take 2 cc. of plasma (or whole blood) in a 25 cc. volumetric flask. Add about 20 cc. of water and then slowly and with stirring add 1 cc. of a freshly prepared 25 per cent solution of metaphosphoric acid. Fill to the mark with water, shake well, and let stand for 10 minutes with occasional shaking. Filter and take 10 cc. of filtrate in a 25 cc. volumetric flask, add 5 cc. of the M/29.25 silver nitrate solution, 5 cc. of 10 per cent magnesium sulfate solution (to facilitate the flocking out of the silver chloride), fill to the mark with water, shake, and let stand for 5 minutes. Filter or centrifuge the liquid and take 20 cc. of the clear solution in a small Erlenmeyer flask, add 4 cc. of the nitrite, citrate, starch solution, and titrate with the M/58.5 potassium iodide solution.

The calculation is as follows: 1.25 (8 − cc. KI used) × $\frac{5}{4}$ × 100, or, simplified, 156 (8 − cc. KI used) = mg. NaCl per 100 cc. of blood or plasma.

This modification makes the admirable micro titration method of McLean and Van Slyke still available, and the procedure for removing the blood proteins is, we think, somewhat simplified. Another point in its favor is that, if chloride determinations are to be done on whole blood it may be unnecessary in some cases to use a separate portion of blood, but the titration may be done on the filtrates obtained in the determination of non-protein nitrogen according to Folin and Denis,[3] or on the filtrates obtained in blood urea determinations according to a method soon to be published from this laboratory. In this case, since 5 cc. of blood are diluted to 50 cc. the above mentioned formula for the calculation does not hold true. If 10 cc. of a non-protein nitrogen or urea nitrogen filtrate were used, the formula would be:

1.25 (8 − cc. KI used) × 100 mg. NaCl per 100 cc. of blood.

[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 491.

Table I shows how perfectly the results by the modified procedure agree with those of the McLean-Van Slyke method.

TABLE I.

Sample.	Dilution.	Old method.		New method.	
		KI	NaCl per 100 cc.	KI	NaCl per 100 cc.
		cc.	*mg.*	*cc.*	*mg.*
Normal human plasma I	2:25	4.02	620	4.02	620
" " " I	2:25	4.04	618	4.00	623
" " " II	2:25	4.15	601	4.15	601
" " " II	2:25	4.15	601	4.17	598
Beef plasma III	2:25	4.22	590	4.20	594
" " III	2:25	4.24	587	4.24	587
" " III	2:25			4.22	590
" " III	5:50			3.25	595
" " III	5:50			3.27	592
Beef whole blood IV	2:25	4.80	500	4.78	503
" " " IV	2:25	4.76	505	4.80	500
" " " IV	5:50			3.98	502*
" " " IV	5:50			3.95	506*
" " " V	2:25	4.98	472	4.98	472
" " " V	2:25	5.00	469	4.96	474
" " " V	5:50			4.20	475**
" " " V	5:50			4.20	475**

* Non-protein nitrogen filtrates.

** Blood urea filtrates.

I am indebted to Professor Folin for his advice and encouragement in this work.

THE FUNCTION OF MUSCULAR TISSUE IN UREA FORMATION.

By RALPH HOAGLAND and C. M. MANSFIELD.

(From the Biochemic Division, Bureau of Animal Industry, United States Department of Agriculture, Washington.)

(Received for publication, July 9, 1917.)

INTRODUCTION.

The formation of urea in the animal body is a question of fundamental importance in protein metabolism. Certain features of the problem have been worked out with some degree of satisfaction, but there is still uncertainty both as to where and how urea is formed in the body and as to the nature of the processes involved. The present status of our knowledge on the subject may be briefly summarized as follows.

The end-products of protein digestion—chiefly amino-acids—are absorbed directly into the circulatory system from the walls of the small intestines, and are then transported to the various body tissues. The body cells select such amino-acids as are needed for their repair or upbuilding, and quantities in excess of these needs are deaminized with the subsequent production of ammonium carbamate and ammonium carbonate, which are transformed into urea, and removed from the blood by the kidneys. The deaminized residue of the amino-acid molecule may then be utilized for the production of energy or it may be transformed into reserve material—glycogen or fat. In case of the break down of the protein constituents of the cell, it is probable that the amino-acids formed undergo changes similar to those which take place in the amino-acids absorbed from the digestive tract.

The important question, so far as the purposes of this paper are concerned, is: Where and by what means is urea formed in the body?

There is abundant proof that the liver performs an important function in urea formation. Satisfactory evidence that it is the sole source, or even the most important source, of urea production in the body is lacking. On the other hand, the prevailing theory is that urea formation is in all probability a function of body cells in general rather than of the liver cells in particular. It seems worth while to quote briefly the views of a few leading physiological chemists on this subject.

Otto von Fürth[1] states: "Im Ganzen neigt man gegenwärtig mehr und mehr der Meinung zu, dass die Fähigkeit, Harnstoff zu bilden, nicht ein Vorrecht der Leber, vielmehr, ebenso wie die Fähigkeit, Eiweiss zu verbrennen, eine der allgemeinen Eigenschaften lebender Zellen ist."

Abderhalden[2] says: "Aus dem vorliegenden Beobachtungen ergibt sich der Schluss, dass die Leber Harnstoff bildet. Der Versuch am überlebenden Organ hat das eindeutig bewiesen. Wahrscheinlich kann er auch von allen anderen Körperzellen erzeugt werden. Allerdings fielen bis jetzt die Versuche an anderen überlebenden Organen als der Leber negativ aus. Die Beobachtungen an Individuen mit pathologisch veränderter Leber und vor allem die Tierversuche, bei denen die Leber künstlich schwer geschädigt wurde, zwingen uns jedoch zu der Annahme, dass die verschiedensten Gewebe Harnstoff bilden können."

Folin[3] states: "The hypothesis that the urea forming process is thus probably largely a matter of muscle metabolism predicates of course nothing as to the nature of the process. In no way does it invalidate or weaken the prevailing view that deaminization rather than oxidation represents the first step in the formation of urea from amino-acid nitrogen. Ascribing to the muscles the greatest share in the urea formation represents, therefore, in no essential point a return to the earlier teachings of Pflüger or of Voit."

Bayliss[4] has this to say concerning the formation of urea in the body: "In the second place, certain experiments tend to show that the liver has not much more power of deamination than the other cells of the organism. The experiments of Lang (1904) and of Miss Bostock (1911) have shown that tissues *in vitro* are capable of deaminating amino-acids to some extent, but that the process is not quite the same as that in the living organism, since

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[1] Von Fürth, O., Probleme der physiologischen und pathologischen Chemie, Leipsic, 1913, ii, 101.

[2] Abderhalden, E., Lehrbuch der physiologischen Chemie, Berlin, 3rd edition, 1914, i, 586.

[3] Folin, O., and Denis, W., Protein metabolism from the standpoint of blood and tissue analysis. Third paper. Further absorption experiments with especial reference to the behavior of creatine and creatinine and to the formation of urea, *J. Biol. Chem.*, 1912, xii, 161.

[4] Bayliss, W. M., Principles of General Physiology, London, 1915, 265.

amides are more readily acted on *in vitro* than are amino-acids, while the contrary is the case in the organism.

"We may conclude that amino-acids are supplied to the tissues, and, with the exception of that small part used for repair or growth, are deaminated there.

"The great activity of the liver in the conversion of ammonia to urea makes it probable that the main part of the ammonia from the tissues is converted into urea in that organ."

The basis for the theory that urea formation is a function of body cells in general rather than of liver cells in particular is, of course, founded upon the idea that the metabolism of the individual cell is complete in itself. Thus, in case of muscle cells, it is well known that they contain several proteolytic enzymes which actively break down the cell proteins with the production of amino-acids and ammonia, if not urea. Hoagland, McBryde, and Powick[5] found that when muscular tissue of an ox was incubated for 100 days under aseptic conditions at 37°C. the amino-acid content of the tissue increased 741 per cent and the ammonia content 519 per cent. While the two constituents increased at somewhat nearly the same rate, yet at the end of the period the amino-acid nitrogen constituted 18.9 per cent of the total nitrogen of the tissue, while the ammonia nitrogen constituted only 1.6 per cent. Unfortunately no study was made of the urea content of the muscular tissue in those experiments. The results of the experiments indicate clearly that muscle cells have to a high degree the ability to break down their protein constituents into amino-acids, and to a lesser degree, into ammonia. The lower production of ammonia was probably due to certain limiting factors which are discussed by the authors of the above paper. These changes are in harmony with the theory that ammonia is an intermediate product in the transformation of amino-acids into urea. Now since muscle cells break down their protein constituents into amino-acids and ammonia, it is reasonable to expect that those cells might transform the ammonia into urea. At any rate, that is the idea which led the writers to undertake the investigations which are to be reported in this paper. Again, it may be that the change is carried no further than ammonia by the muscle cells, and perhaps by other

[5] Hoagland, R., McBryde, C. N., and Powick, W. C., Changes in fresh beef during cold storage above freezing, *U. S. Dept. Agric., Bull. 433*, 1917.

body cells as well, and that the ammonia is carried to the liver where it is transformed into urea. The well known marked ability of the liver to change ammonia into urea harmonizes with this view.

Direct experimental evidence that urea is formed elsewhere in the body than in the liver is comparatively meager. The problem is a difficult one to solve. It necessitates either the complete removal of the liver from the circulation of an animal and a study of urea production in the body under such conditions, the difficulties of such a procedure being obvious; or it requires the identification of urea-forming enzymes in body tissues other than the liver. There is considerable indirect evidence that urea may be formed elsewhere in the body than in the liver, but the value of such evidence is uncertain, and it will not be presented.

The following direct experimental evidence supports the view that urea formation is a function of body cells in general.

Matthews and Nelson[6] conducted experiments in which solutions of amino-acids were injected, in one case into the muscular tissue of dogs with Eck fistulas, and in the second case into dogs from which all the abdominal viscera except the kidneys and a small portion of the liver had been removed. In the first instance there was a slight increase in the ammonia and urea content of the urine after 2 to 2½ hours. In the second instance there was always an increase in the ammonia content of the urine at the end of the 1st hour. The increase in the urea, which generally appeared from 1 to 2 hours later, was not so constant, only being manifest in about 90 per cent of the experiments. In some instances the only result obtained was a marked increase in ammonia accompanied by a decrease in urea.

Fiske and Sumner[7] injected solutions of amino-acids into animals in which the liver and kidneys had been removed from the circulation, and as checks, ran controls in which only the kidneys were removed from the circulation. The experimental period amounted to 3 hours or less. On the whole, it was found that there was an appreciable accumulation of urea both in the blood and the muscular tissue after the injection of amino-acids, and that the accumulation was practically as great when the liver was excluded from the circulation as when it was in its normal relations to the other organs. As a result of their experiments the authors conclude that the liver is not the chief site of urea formation from amino-acids.

With the exception of arginase, urea-forming enzymes have not been identified in body tissues.

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[6] Matthews, S. A., and Nelson, C. F., Metabolic changes in muscular tissue. I. The fate of amino-acid mixtures, *J. Biol. Chem.*, 1914, xix, 229.

[7] Fiske, C. H., and Sumner, J. B., The importance of the liver in urea formation, *J. Biol. Chem.*, 1914, xviii, 285.

EXPERIMENTAL.

The purpose of the experiments which are to be reported in this paper was to determine whether or not urea-forming enzymes are present in muscular tissue.

The general plan of the experiments was to obtain sterile samples of muscular tissue by aseptic methods and incubate the tissue in sterile containers for various periods of time, urea being determined in the fresh tissue and in the incubated samples. The general method of procedure was as follows.

Methods.

Fat steers were slaughtered at a local abattoir by the customary methods under the direction of the authors. The operation was carried on with as great dispatch and under as clean conditions as possible. Prior to slaughter the killing floor was washed with hot water and then with a solution of mercuric chloride (1: 1,000). The animal was stunned, hoisted from the floor, and the large blood vessels of the throat were severed. When bleeding was completed the carcass was dropped on to the clean killing floor and wet down with hot water. The proposed lines of incision for the removal of the hide were first scrubbed with hot water and soap and then with a hot solution of mercuric chloride (1: 1,000). The knives and saws used during slaughter were immersed in hot water and then placed in a hot solution of mercuric chloride from time to time during slaughter. One hind quarter from each animal slaughtered was selected for experimental purposes. The hide was removed from this quarter with as great care as possible to avoid contamination of the freshly exposed tissues. During the removal of the hide, cheese-cloth that had previously been soaked in mercuric chloride solution was wrapped around the surface of the hind quarter as rapidly as the hide was removed; and finally when it had been separated from the carcass, the quarter of beef was wrapped with dry cheese-cloth and paper and immediately and rapidly transported to the laboratory. At the laboratory the outer coverings were removed from the quarter of beef, and it was placed in a special room where samples of the muscular tissue were taken for incubation and analysis.

Method of Taking Samples.

The room in which the samples of muscular tissue were taken was about 10 ft. square and provided with a false cheese-cloth ceiling at a height of 10 ft. A window which opened into the room was fitted with a cheese-cloth screen. Prior to taking samples the room was thoroughly washed and then sprayed with a 3 per cent solution of liquor cresolis compositus.

The operators wore sterile gowns, head cloths, and rubber gloves while taking the samples.

The bichloride cheese-cloth was first removed from the quarter of beef and the exposed surface was then wet down with a solution of bichloride of mercury and wiped dry with a sterile cloth. The proposed lines of incision were sterilized by means of a large steel spatula previously heated to a bright red color. Sterile knives and forceps were used in taking all samples. The seared lines were first cut through to a depth of about 0.5 cm. and a second knife was used to trim back sufficient surface tissue to expose the area of muscular tissue necessary in order to get a sample of the desired size. A third knife was used to cut out a block of muscular tissue, which was immediately taken up by means of a pair of forceps and placed in a sterile glass covered dish; additional samples were taken in a similar manner. Every possible precaution was taken to prevent contamination of the samples taken for incubation.

The dishes in which the samples were incubated are 10 cm. high and 10 cm. in diameter and are provided with loosely fitting glass covers. They are of the type known as "dressing jars." As soon as the desired number of samples had been obtained the dishes were sealed by means of strips of adhesive tape, which were then painted with melted paraffin so as to make the seal practically air-tight. The dishes were weighed empty, after the introduction of the samples of tissue, and at the end of the incubation periods. The dishes were placed in an incubator and held there at 37°C. for various periods of time.

The samples of muscular tissue taken for study were secured from the following muscles: biceps femoris, semimembranosus, semitendinosus, and vastus externus.

Bacteriological Examination of Samples.

The incubated samples were examined from time to time and those which showed apparent evidences of bacterial contamination were discarded. Samples which appeared to be sterile were selected at intervals and subjected to a careful bacteriological examination to determine whether bacteria were present or not. Chemical results are reported only on samples proven to be free from bacteria. The following procedure was employed in the bacteriological examination of the samples.

The tape was first removed from the jar and the edges of the cover were flamed. Since considerable quantities of juice had exuded from the samples of muscular tissue during incubation, the juice as well as the tissue was examined for the presence of bacteria. 0.5, 0.25, and 0.1 cc. portions of the juice were plated with standard agar. Portions of tissue were first taken from the surface of the sample, it was then cut in two, and similar cultures were taken from the interior, sterile knives and forceps being used. Three portions were taken from the surface and a like number from the interior. One portion from each surface was placed in peptonized beef

broth for the development of aerobic bacteria and two portions were introduced into tubes of glucose agar for the development of anaerobic organisms. The tubes of agar had first been boiled to drive off traces of air and then allowed to cool to approximately 40°C. The portion of tissue was introduced into the tube and as soon as the material had settled to the bottom the tube was placed in ice water for the agar to harden so as to exclude air and encourage the growth of anaerobic organisms. Smears were also made on cover glasses and examined for bacteria. All cultures were incubated for at least 6 days, and in the absence of growth the samples of muscular tissue were accepted as being sterile.

Methods of Analysis.

The muscular tissue was freed as far as practicable from visible fat and connective tissue, and finely ground in a meat grinder. In case of the incubated samples, from which considerable juice had exuded, the tissue was first ground and then thoroughly mixed with the juice. Analytical work was always started immediately after the sample had been prepared for analysis.

Urea was determined by the well known urease method. Duplicate determinations were made on all samples, and the average result is reported. The procedure employed was as follows.

25 gm. of the ground tissue were introduced into a 200 cc. Erlenmeyer flask, and 100 cc. of absolute alcohol added. The flask was stoppered and shaken to break up lumps of tissue and the shaking was repeated at intervals during an extraction period of about 20 hours. The alcoholic extract was then decanted into a beaker and the tissue residue was transferred to a mortar and ground in the presence of sand. The ground tissue and alcoholic extract were returned to the flask and extraction was continued for 2 to 3 hours or longer with occasional shakings. The contents of the flask were then filtered on an asbestos filter with the aid of suction, and the flask and filter were washed with hot 95 per cent alcohol. The filtrate was transferred to a beaker and evaporated to about 10 cc. on a steam bath, but never to dryness. The contents of the beaker were transferred to a 25 cc. volumetric flask and made to volume.

A 10 per cent solution of a dry commercial preparation of urease containing the proper quantity of mixed phosphates to insure the maximum activity of the enzyme was made up just before each set of determinations was to be made.

The apparatus used for the determination of urea and ammonia in the extracts of the tissue is of the same general character as that commonly used for the determination of ammonia by the Folin aeration method, and it need not be described in detail. Test-tubes 6½ inches long and 1¼ inches

in diameter and having a capacity of about 100 cc. were used to hold the tissue extract.

25 cc. of N/50 sulfuric acid, three drops of octyl alcohol, 7 drops of a 0.05 per cent solution of methyl red, and 50 cc. of water were introduced into each absorption cylinder. 10 cc. of the concentrated tissue extract were introduced into each of two 100 cc. test-tubes, one portion for the determination of ammonia, the other for urea and ammonia combined. To the tube containing the first portion were added 15 cc. of water and 7 drops of octyl alcohol, and to the second tube were added 2 cc. of a 10 per cent solution of urease, 7 drops of octyl alcohol, and 13 cc. of water. The tubes were connected with the apparatus and allowed to stand 45 minutes for the urease to convert the urea into ammonia, when air was drawn through the apparatus at a moderate rate for 3 to 5 minutes. Suction was then turned off, the test-tubes were disconnected, and 12 gm. of potassium carbonate were added to each tube containing tissue extract. The tubes were immediately connected with the apparatus and air was drawn through it, slowly at first, then as rapidly as practicable, for a total period of at least an hour. The excess of acid in the absorption cylinders was titrated against N/50 NaOH.

In making calculations correction was made for the ammonia liberated from the aliquot portion of the tissue extract which had not been treated with urease. The ammonia liberated from urea by the action of urease was calculated in terms of percentages of urea in the original tissue. In cases where the samples of muscular tissue had lost weight during incubation, the data have been corrected for that loss. Following the procedure which has been described it was found that when known quantities of urea were added to samples of muscular tissue the amount present could be determined with a high degree of accuracy.

Experiment 1.—A fat steer was slaughtered at a local abattoir and one hind quarter was transported to the laboratory, the procedure requiring 1 hour and 25 minutes. Samples were taken for incubation from the following muscles: biceps femoris 11, semimembranosus 9, vastus externus 7, and semitendinosus 3, total 30. The weights of the samples ranged from 90 to 409 gm., the average weight being 226 gm. Such large samples were taken because a study was being made not only of possible urea-forming enzymes but of other muscle enzymes as well. Samples were also taken from each muscle for the determination of urea in the fresh tissue. The samples taken for incubation were placed in an incubator at 37°C. 8 hours and 20 minutes after the death of the animal. The analyses of the fresh tissue were started 7½ hours after the animal was killed. The incubated samples were examined macroscopically at the end of 24, 48, and 72 hours, and subsequently at less frequent inter-

vals. Samples showing positive evidences of contamination were discarded. Usually, though not always, samples which showed no macroscopic evidence of bacterial contamination after incubation for 7 days would prove to be sterile upon careful bacteriological examination. A total of 7 sterile samples, or 23.3 per cent of the samples taken, was obtained from this quarter of beef. While this seems like a rather small percentage of sterile samples, yet every possible precaution was taken to avoid contamination of the samples taken. The difficulties attending the operation of securing sterile samples of muscular tissue weighing several hundred gm. by aseptic methods can be appreciated only by one who has undertaken such an experiment. The changes in the urea content of the muscular tissue during incubation for various periods of time are shown in Table I. "Incubation period" as used in this and following tables denotes the time which elapsed between the death of the animal and the starting of the analysis.

TABLE I.

Serial No.	Samples.	Incubation period.		Urea.
		days	*hrs.*	*per cent*
7	Biceps femoris.		7½	0.0180
11	" "	2	3	0.0201
12	" "	3	4	0.0226
13	" "	8	2	0.0241
14	" "	15	2	0.0206
15	" "	22	3	0.0262
8	Vastus externus.		7½	0.0176
16	" "	29	3	0.0172

Experiment 2.—In this experiment samples of tissue were taken from the biceps femoris and semimembranosus muscle, in this instance about 30 minutes after the death of the animal, and the urea determinations were started at the abattoir. The analyses were started about 70 minutes after the animal was killed. At the laboratory additional samples were taken from the above named muscles for incubation. 16 samples were taken from the biceps femoris and 6 from the semimembranosus muscles, a total of 22. The weights of the samples ranged from 124 to 323 gm., the average weight being 224 gm. The samples were incubated at 37°C. for periods ranging from 5½ hours to 42 days. Of these samples, 7 from the biceps femoris muscle were found to be sterile, or 31.8 per cent of the total number of samples taken. On account of lack of material for all the determinations which were being made, urea was determined in only 4 of the 7 sterile samples. The urea contents of the fresh and incubated samples of muscular tissue are shown in Table II.

TABLE II.

Serial No.	Samples.	Incubation period.	Urea.
			per cent
18	Biceps femoris.	1 hr. 10 min.	0.0165
23	" "	3 days. 2 hrs.	0.0153
28	" "	27 " 1 hr.	0.0180
39	" "	34 " 3 hrs.	0.0178
41	" "	41 " 3 "	0.0153

Experiment 3.—In this experiment also the urea determinations were started at the abattoir. At the laboratory additional samples were taken from the biceps femoris and semimembranosus muscles for analysis and incubation. With one exception, samples of tissue were taken for analyses at 2 hour intervals up to 12 hours from the time the animal was killed. Subsequently samples were analyzed at less frequent intervals. Samples of tissue that were selected for analysis 2 and 4 hours after slaughter were taken direct from the quarter of beef and were not subjected to bacteriological examination, since there had been no opportunity for bacterial penetration of the tissue. A total of 13 samples was taken for incubation, 6 from the biceps femoris and 7 from the semimembranosus muscles. Six of the samples proved to be sterile, or 46 per cent of the total number of samples incubated. Urea determinations were made on only 4 of the 6 sterile incubated samples. The urea contents of the fresh and incubated samples of muscular tissue are shown in Table III.

TABLE III.

Serial No.	Samples.	Incubation period.	Urea.
			per cent
29	Biceps femoris.	1 hr.	0.0156
31	" "	2 hrs. 45 min.	0.0156
32	" "	4 " 15 "	0.0161
33	" "	6 " 20 "	0.0155
35	" "	10 " 30 "	0.0159
36	" "	2 days. 3 hrs.	0.0142
29	Semimembranosus.	1 hr.	0.0161
46	"	20 days. 4 hrs.	0.0163

DISCUSSION OF RESULTS.

In Experiment 1, as shown by the data presented in Table I, there is apparently a slight increase in the urea content of the biceps femoris muscle on incubation for periods varying from 2

days and 3 hours to 22 days and 3 hours. The increases do not proceed at a regular rate, however, the sample incubated 15 days and 3 hours containing practically the same amount of urea as the sample incubated 2 days and 3 hours. The vastus externus muscle shows practically the same urea content after incubation for 29 days and 3 hours as that of the fresh tissue. On the whole, these data do not indicate that the samples of muscular tissue examined had any appreciable ability to form urea.

In Experiment 2, as indicated by the results shown in Table II, there were only slight changes in the urea content of samples of the biceps femoris muscle incubated for periods ranging from 3 days and 2 hours to 41 days and 3 hours. These changes are irregular in character and are probably due to experimental error. These data do not indicate that the muscular tissue examined had any ability to form urea.

The data presented in Table III, Experiment 3, show practically no change in the urea content of samples of the biceps femoris muscle incubated for periods ranging from 2 hours and 45 minutes to 2 days and 3 hours. The slight changes are practically within the limit of experimental error. These data are of particular interest in showing practically no change in the urea content of the muscle either prior to rigor mortis or subsequently. There was no change in the urea content of the semimembranosus muscle after incubation for 20 days and 4 hours. The data presented in this table indicate that the muscular tissue examined lacked the property of converting its protein constituents into urea.

On considering the results of the experiments as a whole, it may be well to point out the extent to which the precursors of urea, amino-acids and ammonia, accumulate in muscular tissue during aseptic autolysis. These constituents were not determined in the experiments here reported, since Hoagland, McBryde, and Powick[5] had previousy reported a study of the changes in the amino-acid nitrogen and ammoniacal nitrogen content of muscular tissue during aseptic autolysis. Table IV has been prepared from data presented in the report by the above authors and it indicates not only the changes in the ammoniacal nitrogen and amino-acid nitrogen content of the muscular tissue during autolysis, but also the possible urea value of the sum of the two constituents.

TABLE IV.

Composition Expressed in Terms of Percentages of Fresh Material.

Serial No.	Incubation period.	Ammoniacal nitrogen.	Amino-acid nitrogen.	Possible urea.
	days			
109	1	0.0087	0.0782	0.1867
110	8	0.0185	0.1412	0.3430
111	15	0.0225	0.1931	0.4631
112	22	0.0254	0.2101	0.5059
113	29	0.0349	0.4232	0.9840
120	43	0.0365	0.4641	1.0750
121	65	0.0509	0.4715	1.1230
122	78	0.0476	0.6100	1.4125
124	94	0.0588	0.6208	1.4598
125	101	0.0629	0.7658	1.7800

The results presented in Table IV are of interest in showing the marked accumulation of the precursors of urea in muscular tissue autolyzed for various periods of time as compared with the urea content of other samples of muscular tissue autolyzed for similar periods, as shown in Tables I, II, and III. For example, in Table II the biceps femoris muscle contained 0.0165 per cent urea 70 minutes after the death of the animal, while a sample of the same muscle incubated for 41 days at 37°C. contained 0.0153 per cent urea, indicating practically no change in the urea content of the tissue. On the other hand, by referring to Table IV it will be seen that the sample of muscular tissue that had been incubated for 43 days contained 1.075 per cent of the precursors of urea expressed in terms of that compound. Thus in case of the second sample the amino-acid and ammoniacal nitrogen, expressed in terms of urea, amounted to 70 times the actual urea content of the sample incubated 41 days. Without going into a detailed discussion of the relations between the amino-acid and ammoniacal nitrogen content of the autolyzed samples of muscular tissue as shown in Table IV, and the urea content of other samples of tissue autolyzed for similar periods, as indicated in Tables I, II, and III, the fact that there is a marked increase in the precursors of urea in the muscular tissue, but that there is practically no change in the actual urea content of the tissue, indicates that the muscular tissue, of itself, lacks the ability to form urea. The proof is not absolute, of course, but it is highly presumptive.

In this connection it should be mentioned that the enzyme arginase discovered by Kossel and Dakin[8] cannot be regarded as an important urea-forming agent in muscular tissue since the above authors reported the enzyme to be present only in traces in that tissue, while Mathews[9] states that arginase is absent from muscular tissue.

SUMMARY.

Taken as a whole, the results of the experiments reported in this paper tend to show that urea formation is not an important function of muscular tissue. It must be admitted that these findings are contrary to what was expected, and to the more or less generally accepted theory that urea formation is a function of body cells in general; but no other interpretation of the facts seems possible.

In view of the well established fact that the liver plays an important part in the formation of urea in the body, these findings lend support to the view that urea production is chiefly a function of that organ.

[8] Kossel, A., and Dakin, H. D., Weitere Untersuchungen über fermentative Harnstoffbildung, *Z. physiol. Chem.*, 1904, xlii, 184.

[9] Mathews, A. P., Physiological Chemistry, New York, 2nd edition, 1916, 669.

GLYCOLYTIC PROPERTIES OF MUSCULAR TISSUE.

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INTRODUCTION.

Our knowledge concerning the processes by which carbohydrates are utilized by the animal body is very unsatisfactory. It may be safely assumed, however, that the entire cycle of changes which sugars undergo after absorption into the circulatory system of the body is due to the action of enzymes; but experimental evidence in support of this assumption is very incomplete. Since the discovery of zymase by Buchner (1) there is no good reason for thinking that the sugar-oxidizing properties of the body are intimately bound up with the protoplasm of the living cell, any more than that alcoholic fermentation is dependent upon the living yeast plant.

A large amount of experimental work has been done in an attempt to identify glycolytic enzymes in muscular and other body tissues, and in the blood as well. The question as to whether the pancreas secretes a coenzyme or activator necessary for the activity of the glycolytic enzyme of the other body tissues has also been the subject of a great deal of study. Unfortunately the present state of our knowledge concerning the subject is very much confused. A considerable amount of work has been done which apparently shows that muscular and certain other body tissues, entirely apart from any secretion of the pancreas, have the property of oxidizing sugars. On the other hand, a considerable number of workers report results which apparently indicate that muscular and other body tissues, either singly or in combination with extracts from the pancreas, have no glycolytic properties whatever. A third group of workers claims that muscular tissue of itself has but very slight or no glycolytic properties, but that in the

presence of an activator supplied by the pancreas glycolysis takes place. The evidence is so conflicting and confusing that it is useless to discuss the work which has been done in any detail in an attempt to arrive at the truth of the matter. It does seem worth while, however, to present the names of the more important investigators of this subject.

The most ardent supporter of the theory that muscular and other body tissues contain enzymes which, apart from any coenzyme or activator supplied by the pancreas, oxidize sugar is Stoklasa (2–6), who has contributed a large amount of apparently very painstaking work on the subject. His work has been confirmed, in part at least, by that of Blumenthal (7), Brunton and Rhodes (8), Feinschmidt (9), Nanking (10), and Ransom (11).

Cohnheim (12–15), on the other hand, in his earlier work held that muscular and other body tissues had but very slight or no glycolytic properties of themselves, but that in the presence of an activator supplied by the pancreas glycolysis takes place. In his later work he modifies his earlier views somewhat by stating that body tissues are not always without glycolytic properties, but that these properties vary greatly. He still insists that the glycolytic properties of the tissues are greatly stimulated by the addition of an extract of the pancreas. His work is supported by that of Hirsch (16), Arnheim and Rosenbaum (17), Sehrt (18), and Hall (19).

Claus and Embden (20, 21) were unable to confirm the results obtained by Cohnheim, and found that muscular tissue of itself had practically no glycolytic properties. Similar results were obtained by McGuigan (22), Simpson (23), Harden and MacLean (24), and Levene and Meyer (25).

The conflicting nature of our knowledge concerning the behavior of sugars in the animal body simply emphasizes the importance of carrying on investigations in such a manner as will furnish exact and incontrovertible evidence on the problem. Clearly the most important point, and the one which should be established first, is: Do muscular and other body tissues of normal animals possess active glycolytic properties, entirely apart from the action of the secretion of any internal organ? That is the problem with which this paper is concerned.

EXPERIMENTAL.

I.

The general plan of the work was to secure sterile samples of muscular tissue from cattle immediately after slaughter, using aseptic methods, and to incubate the pieces of tissue in sterile

containers for various periods of time. The glycogen and the dextrose contents of the tissue were determined as soon as possible after the death of the animal and at intervals thereafter. A study was also made to determine the nature of the end-products of the changes in the carbohydrates of the tissue.

Method of Procedure.

The methods employed in the slaughter of the animals, the taking of samples of tissue for incubation, and the bacteriological examination of the incubated samples are essentially the same as those described in a previous paper (26) by the authors.

Methods of Analysis.

The muscular tissue was freed as far as practicable from visible fat and connective tissue and finely ground in a meat grinder. In case of the incubated samples, from which considerable juice had exuded, the tissue was first ground and then thoroughly mixed with the juice. Analyses were always started immediately after the preparation of the samples.

Glycogen was determined essentially according to the method of Pflüger as described by Abderhalden (27). The purified glycogen was inverted and dextrose was determined by means of Fehling's solution, reduced copper being estimated by Low's copper iodide method. Single determinations were run in the separation of the glycogen from the tissue, but duplicate estimations were made of dextrose in the inverted glycogen solution. Average results are reported in terms of dextrose.

Dextrose was determined by a method previously described by Hoagland (28). Experience has proven the method to yield accurate results. The possibility that a part of the glycogen might be converted into dextrose during the extraction of the sugar from the tissue and the subsequent concentration of the extract was recognized. In order to guard against such a change an excess of calcium carbonate was added to the tissue on extraction, and also to the extract on concentration. The concentrated extract was filtered from the lime residue which was washed free of sugar. In order to determine whether this procedure prevented any conversion of glycogen into dextrose, known quantities of glyco-

gen were added to like portions of a sample of muscular tissue, while no glycogen was added to a similar portion of the same sample, and dextrose was determined in each portion. It was found that the presence of glycogen was without effect upon the dextrose content of the samples examined.

That there might be an appreciable variation in the carbohydrate content of different parts of the same muscle was recognized as another possible source of error. In order to secure information on this point the biceps femoris muscle was dissected out from a hind quarter of beef and freed from surface fat and connective tissue. The muscle weighed 7,420 gm. Sugar was determined in five samples of muscular tissue taken from various parts of the muscle with the following results: 0.228, 0.256, 0.264, 0.255, 0.220 per cent, averaging 0.245 per cent.

From these data it will be seen that the maximum variation amounts to 0.044 per cent, while the greatest variation from the average is 0.025 per cent.

Experiment 1.—A fat steer was slaughtered at a local abattoir, and one hind quarter was transported to the laboratory, the procedure requiring 1 hour and 25 minutes. Samples of tissue were taken from the following muscles: biceps femoris 11, semimembranosus 9, vastus externus 7, and semitendinosus 3, a total of 30. The weights of the samples varied from 90 to 409 gm., the average weight being 226 gm. Portions were taken from each muscle for the determination of glycogen and dextrose in the fresh tissue. The samples taken for incubation were placed in an incubator at 37°C. 8 hours and 20 minutes after the death of the animal. The analyses of the fresh tissue were started 7½ hours after the animal was killed. The incubated samples were examined macroscopically at the end of 24, 48, and 72 hours and subsequently at less frequent intervals. Samples showing positive evidences of contamination were discarded, while those which appeared to be sterile were subjected to careful bacteriological examination. In this and subsequent experiments, unless otherwise stated, analyses are reported only on those incubated samples which were found to be sterile. A total of 7 sterile samples, or 23.3 per cent of the number of samples incubated, was obtained from this quarter of beef. The changes in the glycogen, dextrose, and total carbohydrate content of the samples of muscular tissue incubated for various periods of time are shown in Table I.

Experiment 2.—In this experiment samples of tissue were taken about 30 minutes after the death of the animal, from the biceps femoris and semimembranosus muscles, and glycogen and dextrose determinations were started at the abattoir about 70 minutes after the animal was killed. The quarter of beef was transported to the laboratory where additional samples were taken from the above named muscles for incubation and analysis.

TABLE I.*

Serial No.	Samples.	Incubation period.		Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
		days	*hrs.*	*per cent*	*per cent*	*per cent*	*per cent*
7	Biceps femoris muscle.		7½	0.064	0.163	0.227	
11	" " "	2	3	Trace.	0.239	0.239	+5.3
12	" " "	3	4	"	0.180	0.180	−20.7
13	" " "	8	2		0.186	0.186	−18.1
14	" " "	15	2		0.179	0.179	−21.1
15	" " "	22	3		0.490	0.490	+116.0
8	Vastus externus "		7½	0.018	0.159	0.177	
16	" " "	29	3		0.263	0.263	+48.6
17	" " "	36	6		0.157	0.157	−11.3

* "Incubation period," as used in this and the three tables following, denotes the time which elapsed between the death of the animal and the starting of the analysis.

Sixteen samples were taken from the biceps femoris and 6 from the semimembranosus muscles, a total of 22. The weights of the samples varied from 124 to 323 gm., the average weight being 224 gm. The samples were placed in an incubator 5½ hours after slaughter and were held there at 37°C. for periods ranging from 5½ hours to 41 days. Seven samples from the biceps femoris muscle were found to be sterile after incubation, or 31.8 per cent of the number of samples incubated. Cultures were not taken from the sample analyzed 5½ hours after the death of the animal since there had been no opportunity for bacterial invasion of the tissues. The glycogen, dextrose, and total carbohydrate content of the fresh and incubated samples are shown in Table II.

Experiment 3.—Samples of tissue were taken for analysis immediately after the death of the animal and at approximately 2 hour intervals up to 10 hours from the time the animal was killed, and subsequently at less frequent intervals. Samples taken for analysis 2 and 4 hours after slaughter were not subjected to bacteriological examination since there had been no opportunity for bacterial penetration of the tissues. A total of 13 samples was taken for incubation, 6 from the biceps femoris and 7 from the semimembranosus muscles. The samples were placed in an incubator at 37°C. 5½ hours after slaughter. Six samples were found to be sterile after incubation, or 46 per cent of the number incubated. Three of the sterile samples, Nos. 36, 46, and 47, were incubated in an atmosphere of carbon dioxide. The glycogen, dextrose, and total carbohydrate content of the fresh and incubated samples of muscular tissue are shown in Table III.

TABLE II.

Serial No.	Samples.	Incubation period.	Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
			per cent	*per cent*	*per cent*	*per cent*
18	Biceps femoris muscle.	70 min.	0.274	0.08	0.354	
20	" " "	5½ hrs.	0.014	0.051	0.065	−81.1
21	" " "	10½ "	0.017	0.132	0.149	−57.9
22	" " "	2 days. 1 "		0.158	0.158	−55.4
23	" " "	3 " 2 "		0.155	0.155	−56.2
28	" " "	27 " 1 "		0.030	0.030	−91.5
39	" " "	34 " 3 "		0.062	0.062	−82.5

TABLE III.

Serial No.	Samples.	Incubation period.	Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
			per cent	*per cent*	*per cent*	*per cent*
29	Biceps femoris muscle.	1 hr.	0.672	0.132	0.804	
31	" " "	2 hrs. 45 min.	0.930	0.139	1.069	+33.0
32	" " "	4 " 15 "	0.843	0.133	0.976	+21.4
33	" " "	6 " 20 "	0.731	0.170	0.901	+12.0
34	" " "	8 " 30 "	0.417	0.294	0.711	−11.6
35	" " "	10 " 30 "	0.200	0.322	0.522	−35.0
36	" " "	27 "	0.267	0.447	0.714	−11.1
30	Semimembranosus "	1 "	1.015	0.138	1.153	
46	" "	20 days 4 hrs.		0.462	0.462	−60.0
47	" "	28 " 3 "		0.466	0.466	−59.6

Experiment 4.—Samples were taken for analysis as in Experiment 3. Those taken for analysis 3, 4½, and 6½ hours after slaughter were not subjected to bacteriological examination since there had been no opportunity for bacterial penetration of the tissues. Fourteen samples were taken for incubation, 7 from the biceps femoris and 7 from the semimembranosus muscles. The samples were placed in an incubator at 37°C. 6 hours after slaughter. Three of these samples, or 21.4 per cent of the number incubated, were found to be sterile. Cultures from one sample, No. 56, showed

no growth for 6 days after incubation, but later a slow-growing anaerobic organism developed. The analysis of this sample will be reported in Table IV since it is believed that there must have been but very slight development of the organism in the sample of muscular tissue during the short period of incubation to which it was subjected, a total of 8 hours and 20 minutes. The sterile samples were all obtained from the biceps femoris muscle. The changes in the glycogen, dextrose, and total carbohydrate content of the samples of muscular tissue are shown in Table IV.

TABLE IV.

Serial No.	Samples.	Incubation period.	Glycogen calculated as dextrose.	Dextrose.	Glycogen and dextrose calculated as dextrose.	Change in total carbohydrates.
			per cent	*per cent*	*per cent*	*per cent*
50	Biceps femoris muscle.	1 hr. 10 min.	0.734	0.170	0.904	
52	" " "	3 hrs.	0.564	0.171	0.735	−18.7
53	" " "	4 " 30 "	0.664	0.208	0.872	− 3.5
54	" " "	6 " 30 "	0.430	0.134	0.564	−37.6
*56	" " "	8 " 20 "	0.298	0.362	0.660	−27.0
57	" " "	10 " 30 "	0.148	0.437	0.585	−35.3
58	" " "	12 " 15 "	0.172	0.312	0.484	−46.4
61	" " "	2 days 1 hr.	0.097	0.464	0.561	−37.9
64	" " "	7 " 5 hrs.		0.379	0.363	−59.8
51	Semimembranosus "	1 hr. 10 min.	0.978	0.153	1.131	
55	" "	6 hrs. 30 "	0.257	0.386	0.643	−43.1

* Cultures show slow-growing anaerobic organism after 6 days.

DISCUSSION OF RESULTS.

The experiments will first be discussed separately, and then as a whole, in order to make clear the significance of the results obtained.

Experiment 1.—In this experiment the importance of determining the carbohydrate content of the muscles immediately after the death of the animal was not fully appreciated, so the first analysis was not started until $7\frac{1}{2}$ hours after slaughter. It does not seem worth while to pay particular attention to the changes in the glycogen or the dextrose content of the muscles, but rather to the changes in the sum of the two constituents expressed in terms

of dextrose. These changes are shown most clearly in the column headed "change in total carbohydrates," where the changes are expressed in percentage of the amount of carbohydrates present in the first sample of each muscle analyzed.

The sample of biceps femoris muscle incubated 2 days and 3 hours shows an apparent increase in total carbohydrates amounting to 5.3 per cent, which is practically within the limit of experimental error, while the samples incubated for periods ranging from 3 days and 4 hours to 15 days and 2 hours show decreases in total carbohydrates amounting to approximately 20 per cent. The sample of this muscle incubated 22 days and 3 hours shows an increase in carbohydrates amounting to 116 per cent. The vastus externus muscle shows an increase of 48.6 per cent in total carbohydrates after incubation for 29 days and 3 hours, which confirms the increase in the carbohydrate content of the biceps femoris muscle. The sample incubated 36 days and 6 hours contains 11.3 per cent less carbohydrates than the first sample of this muscle analyzed.

Taken as a whole, the results of this experiment show (1) samples of tissue from both the muscles examined had appreciable glycolytic properties, the decreases in total carbohydrates varying from 11.3 to 21.1 per cent; (2) samples from both muscles apparently had the ability to synthesize carbohydrates.

Experiment 2.—The results obtained in the first experiment suggested the idea that in all probability the glycolytic properties of muscular tissue are most marked for a short time after death, and that these activities are brought to a close by the development of certain conditions associated with rigor mortis. For this reason samples of muscular tissue were analyzed as soon as possible after the death of the animal, in this case 70 minutes, and later at the end of $5\frac{1}{2}$ and $10\frac{1}{2}$ hours. In this experiment the glycogen was very rapidly transformed into dextrose, the change being practically complete at the end of $5\frac{1}{2}$ hours.

The changes in the total carbohydrate content of the samples of muscle during incubation are of great interest. All of the samples, which were incubated for periods ranging from $5\frac{1}{2}$ hours to 34 days and 3 hours, show large relative decreases in total carbohydrates, varying from 55.4 per cent in the sample incubated 2 days and 1 hour to 91.5 per cent in the sample incubated 27 days

and 1 hour. The sample incubated only 5½ hours shows a decrease of 81.1 per cent.

By referring to Table II the column headed "glycogen and dextrose calculated as dextrose," it will be noted that there is first a large decrease in the total carbohydrate content of the sample incubated 5½ hours; while in the sample incubated 10½ hours there is a considerable increase as compared with the 5½ hour sample, but still a marked decrease as compared with the first sample analyzed. This increase in the carbohydrate content of the sample incubated 10½ hours, as compared with the sample incubated 5½ hours, confirms a similar increase observed in Experiment 1. Finally the samples of biceps femoris muscle incubated for 27 days and 1 hour, and 34 days and 3 hours contain less carbohydrates than the sample incubated 5½ hours.

On the whole, the results of this experiment show that the samples of muscular tissue examined had very marked glycolytic properties. It is of particular significance that glycolysis took place so rapidly immediately after the death of the animal. The fact that there was a decrease of 81.1 per cent in the carbohydrate content of the sample of muscular tissue analyzed 5½ hours after the animal was killed, as compared with the amount present in the muscle 1 hour and 10 minutes after slaughter, indicates the rapidity with which the change took place.

Experiment 3.—The importance of analyzing the samples of tissue at frequent intervals following the death of the animal is at once apparent from an examination of the data presented in Table III. The changes in the glycogen content of the samples of the biceps femoris muscle during incubation are of particular interest. The sample analyzed 1 hour after the death of the animal contains 0.672 per cent glycogen, while the one analyzed 1 hour and 45 minutes later, or 2 hours and 45 minutes after the animal was killed, contains 0.930 per cent, or a relative increase of 38.4 per cent over the amount present in the first sample. The samples incubated for 4 hours and 15 minutes, and 6 hours and 20 minutes show gradually decreasing percentages of glycogen as compared with the sample incubated 2 hours and 45 minutes, but, on the other hand, larger percentages of glycogen than the sample analyzed 1 hour after slaughter. The samples incubated 10 hours and 30 minutes, and 27 hours contain less glycogen than

any of the others. These facts are of much importance in that they indicate that muscular tissue has the ability to synthesize glycogen. Since there was no decrease in the dextrose content of the muscle during the increase in glycogen, and because proof is lacking that glycogen can be formed from fat, it appears that the glycogen must have been formed from proteins.

The total carbohydrate content of the biceps femoris muscle shows, first, a relative increase of 33 per cent in the sample incubated 2 hours and 45 minutes as compared with the sample incubated 1 hour, and then increases of 21.4 and 12.0 per cent in the samples incubated 4 hours and 15 minutes and 6 hours and 20 minutes, respectively. The samples incubated for periods ranging from 8 hours and 30 minutes to 27 hours show decreases varying from 11.1 to 35 per cent. If the percentage changes in the total carbohydrate content of the samples of muscle be referred to the amount present in the sample incubated 2 hours and 45 minutes as a basis, it will be found that the samples incubated for periods ranging from 4 hours and 15 minutes to 27 hours show the following decreases in carbohydrates: 8.7, 15.7, 33.5, 51.0, and 33 per cent.

Unfortunately samples of the semimembranosus muscle were not analyzed at as frequent intervals as were the samples of the biceps femoris muscle. The samples of the semimembranosus muscle incubated for periods of 20 days and 4 hours and 28 days and 3 hours show decreases in total carbohydrates amounting to practically 60 per cent of the amount present in the sample analyzed 1 hour after slaughter. Judging by the results obtained in the other experiments, it is probable that the decreases in the carbohydrate content of the muscle just noted took place within a few hours after the death of the animal.

Taken as a whole, the results of this experiment indicate (1) samples of the biceps femoris muscle apparently had the ability to synthesize glycogen and at the same time to increase their content of total carbohydrates; (2) samples of the biceps femoris muscle showed marked glycolytic properties, over 50 per cent of the carbohydrates disappearing within a period of 8 hours; (3) samples of the semimembranosus muscle also had marked glycolytic properties.

Experiment 4.—Samples from both the biceps femoris and

semimembranosus muscles showed appreciable glycolytic properties. The decreases in the total carbohydrate content of the muscles did not proceed at a constant rate, the changes taking place most rapidly during the first 12 hours after the death of the animal. The total carbohydrate content of the biceps femoris muscle had decreased 18.7 per cent 3 hours after the death of the animal, 46.4 per cent after 12 hours and 15 minutes, and 59.8 per cent after 7 days and 5 hours. The semimembranosus muscle decreased 43.1 per cent in total carbohydrates in a period of 6 hours and 30 minutes following slaughter.

Taken as a whole, the results of this experiment show that the samples of muscular tissue examined had appreciable glycolytic properties, the disappearance of sugar taking place most rapidly within 12 hours after the death of the animal. These results confirm those obtained in the previous three experiments.

SUMMARY OF RESULTS.

The results of the experiments which have been reported show that muscular tissue from a normal ox has appreciable glycolytic properties, and that these properties are most active for a short time following the death of the animal. The factors which limit the glycolytic changes which take place in dead muscular tissue under such conditions as prevailed in these experiments have not been determined as yet, but in all probability the changes are limited by conditions associated with the development of rigor mortis.

Muscular tissue also appeared to have the ability to synthesize carbohydrates, both glycogen and dextrose, presumably from proteins.

These findings show that appreciable glycolysis may take place in muscular tissue without the aid of any extract from pancreatic tissue. These results do not, on the other hand, indicate that the pancreas does not play an important part in the glycolysis that takes place in living muscular tissue. In the experiments which have been reported the animals were bled as completely as possible, but the tissues were not washed free from blood, since this procedure did not seem practicable under the conditions of the experiments. It is barely possible, but highly improbable, that the small amount of blood which remained in the muscular tissue

may have supplied an activator or coenzyme previously secreted by the pancreas, which stimulated glycolysis. Rather, the authors are of the opinion that the pancreas functions somewhat differently in sugar metabolism. They are of the opinion that in all probability the pancreas plays an important part in the actual formation of the glycolytic enzymes in living tissues, but that active glycolysis may take place in dead tissues in the absence of any extract of the pancreas.

The possibility that the decrease in the copper-reducing action of the clarified extracts of the incubated samples of muscular tissue might be due to the formation of disaccharides, as suggested by Levene and Meyer (29), has been considered. In a considerable number of cases the copper-reducing action of clarified muscle extracts was determined both before and after inversion, but in no case was there any evidence of the formation of disaccharides.

Attention has been called to the somewhat irregular character of the changes in the carbohydrate content of the muscular tissue during autolysis. In a small degree such irregularities may be due to unavoidable errors, but for the most part they must be due to other causes. Considering the complex nature of the metabolism of sugar in the living organism, it is reasonable to expect that the changes which take place in the carbohydrates of dead muscle may be equally or even more complex. In the normal living organism the various forces work in harmony so as to dispose of the absorbed sugars according to the needs of the body; in the dead tissues the equilibrium of forces has been broken up and other factors enter into play, many of which are not clearly understood, so that the changes which take place under such conditions cannot compare either in orderliness or in extent with those that take place in the living organism.

Glycolysis has been measured in the experiments which have been reported by the disappearance of carbohydrates. The questions naturally arise: What has become of the carbohydrates? Have they been oxidized completely to carbon dioxide and water, as in the living organism, or has the oxidation been incomplete? The work of Stoklasa suggests that the sugar present in the muscular tissue may have been oxidized only to carbon dioxide and alcohol. In order to secure information on this subject the following experiments were conducted.

II.

The purpose of the experiments which are to be reported was to determine whether the glycolysis that takes place in muscular tissue results in the production of carbon dioxide and alcohol.

The general plan of these experiments was to secure sterile samples of muscular tissue by aseptic methods as soon as possible after the death of the animal, and to incubate the tissue in sterile media in suitable fermentation tubes so as to collect any gas that might be formed. Special large size fermentation tubes, of the so called "fish hook" type, having a capacity of 360 cc., were used. Samples of muscular tissue were taken in the special room previously described. Every precaution was taken to avoid contamination of the samples of tissue. A strict bacteriological control was exercised over all the experiments.

Experiment 5.—Fermentation tubes were filled with neutral plain beef broth to which had been added 1 per cent by volume of a solution containing 10 per cent of mixed phosphates (1 part monosodium phosphate, 2 parts disodium phosphate) to preserve neutrality, and which had been saturated with carbon dioxide. The filled fermentation tubes were held 3 days in an incubator at 37°C., and those which showed bacterial contamination were discarded. In this and subsequent experiments the fermentation tubes were removed from the incubator only a few minutes before the samples of tissue were to be placed in the tubes so that the temperature of the media would be close to 37°C.

A rabbit weighing 2,540 gm. was killed by severing the anterior aorta, and when bleeding was complete the carcass was immersed in a solution of mercuric chloride (1:1,000) until the hair was thoroughly wet. The skin was removed with as great care as possible to avoid contamination of the exposed tissues and the carcass was wrapped in a cloth previously soaked in a solution of mercuric chloride. In taking samples of muscular tissue for incubation, the proposed lines of incision were seared by means of a hot spatula, the surface tissue was trimmed back, and a sample of the underlying muscular tissue was taken out by means of a knife and pair of forceps, and transferred to a fermentation tube. A total of 5 samples weighing from 9 to 26 gm. were taken in this way. The first sample was taken 7 minutes after the death of the animal, and the last sample after 33 minutes. The tubes were then placed in an incubator at 37°C.

Immediately after the samples of tissue had been placed in the fermentation tubes it was noticed that a few small gas bubbles had collected on the surfaces of the pieces of tissue. After 1 hour from the time the rabbit was killed a few small bubbles had collected in the tops of the

closed arms of four tubes. After 2, 3, 5, 6, and 24 hours there had been no further production of gas. After incubation for 72 hours, two tubes were found to be contaminated. Since previous experiments have shown that glycolysis takes place most rapidly during the first 12 hours after the death of the animal, the fact that the incubated samples of muscular tissue gave rise to practically no production of gas during the first 24 hours indicates that carbon dioxide was not an end-product of any glycolysis that took place in the tissue. In view of the negative results, the fact that two tubes proved to be contaminated is immaterial.

Experiment 6.—A rabbit weighing 2,590 gm. was killed and skinned as in Experiment 5. The surfaces where the samples of muscular tissue were to be taken were not seared but simply wiped with cotton saturated with a solution of mercuric chloride (1: 1,000). The surface tissue was first trimmed back and a sample of muscular tissue was then taken out by means of a knife and pair of forceps, immersed in a sterile normal salt solution so as to remove mercuric chloride, and transferred to a fermentation tube previously filled with Locke's solution. This solution was made up according to the following formula:

	per cent
Sodium chloride	0.900
Calcium "	0.024
Potassium "	0.042
Dextrose	0.100

The first sample of muscular tissue was taken 15 minutes after the death of the animal, and the last sample 8 minutes later, when the tubes were at once transferred to an incubator at 37°C. Six samples were taken, the weights ranging from 36 to 75 gm.

A few minutes after the pieces of tissue had been placed in the fermentation tubes a few small gas bubbles were observed clinging to the surfaces of the pieces of tissue. After incubation for 1 hour and 4 hours there had been no further production of gas. After 5 hours the gas bubbles had practically all disappeared. At the end of 24 hours 4 tubes proved to be contaminated, and three of these showed abundant gas formation. The two sterile tubes showed no gas production.

Experiment 7.—This experiment was conducted in a similar manner to Experiment 6 and gave like results.

Experiment 8.—In this experiment samples of muscular tissue of an ox were taken by aseptic methods and transferred to fermentation tubes containing Locke's solution. The methods employed in the slaughter of this animal and the taking of the samples of tissue have been described under Experiment 4. The first sample of tissue was transferred to a fermentation tube 2 hours and 42 minutes after the death of the animal, and the last sample 17 minutes later when the tubes were placed in an incubator at 37°C. A total of six samples was taken. The tubes were examined after incubation for 50 minutes, 2½, 5, and 21 hours, and 2 days and 3 hours, but in no case was there any evidence of gas production. After incuba-

tion for 2 days and 3 hours the tubes showed no positive evidences of contamination, but cultures taken from the tubes showed that all samples were contaminated. Since there had been no gas production, the fact that all the tubes were contaminated is immaterial.

DISCUSSION OF RESULTS.

The results of the experiments which have been reported indicate that carbon dioxide is not an end-product of the glycolysis that takes place in muscular tissue autolyzed under aseptic conditions. The few small gas bubbles observed clinging to pieces of muscular tissue in several instances cannot be regarded as being of any significance. Since no carbon dioxide was formed it was unnecessary to test for alcohol. It is evident that the end-products of the glycolysis that took place under the conditions of these experiments are intermediate between dextrose, and carbon dioxide and water. The nature of these products remains to be determined, but our knowledge of the changes that take place in sugars in the living organism indicates very strongly the probable character of these intermediate products. The conditions necessary for the complete oxidation of sugars by dead muscular tissue are not clear.

CONCLUSIONS.

The results of the experiments which have been reported in this paper appear to justify the following conclusions.

1. Muscular tissue autolyzed under aseptic conditions shows appreciable glycolytic properties.

2. Glycolysis takes place most rapidly within a comparatively few hours after the death of the animal.

3. The glycolysis that took place under the conditions of these experiments did not result in the production of carbon dioxide.

4. Muscular tissue apparently has the ability to synthesize carbohydrates.

5. In part at least, and probably in their entirety, the processes by which sugars are utilized by the animal body are enzymatic in nature.

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TABLES FOR FINDING THE ALKALINE RESERVE OF BLOOD SERUM, IN HEALTH AND IN ACIDOSIS, FROM THE TOTAL CO_2 OR THE ALVEOLAR CO_2 OR THE pH AT KNOWN CO_2 TENSION.

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(Received for publication, May 30, 1917.)

The object of the present paper is the comparison of the results obtained by means of the hydrogen electrode and with alveolar CO_2 apparatus with those obtained by means of the Van Slyke apparatus for total CO_2 in plasma. Van Slyke has given us a factor for roughly finding the alveolar CO_2 from the plasma CO_2, but this does not help us interpret the determinations with the hydrogen electrode, and we hope that the following tables may elucidate the data on acidosis and give some basis for analysis of such investigations as those of Peters.

Since the alkaline reserve has been expressed in various units in papers by the senior author it is necessary to state that in the present paper we mean the sum of the equivalents of strong bases minus the sum of the equivalents of strong acid in the serum, expressed as a fraction of a normal solution. It is the excess base or titratable alkalinity, where the precautions described below are taken to insure the correct end-point in the titration, *viz.*, the pH of distilled water at the same CO_2 tension.

It appears to be approximately true that blood serum behaves as a bicarbonate solution made isotonic by the addition of NaCl in regard to pH and CO_2 tension (McClendon, *a*, *b*). That is to say, the non-volatile buffers, phosphates and proteins, do not have an easily measurable effect on the pH at 42 mm. CO_2 tension. The concentration of diffusible phosphates is about 0.001 M in serum, and failure to detect their effect on the pH is due to

their low concentration. The concentration of protein is less in serum than in plasma, but the sodium oxalate necessary to prevent coagulation of plasma is reduced to alkali by ashing in the compensation dialysis method of determining the alkaline reserve (*i.e.*, dialyzing serum against $NaHCO_3$ solutions made isotonic with NaCl and finding the solution not changed by dialysis). Hence we confined our studies to serum, in order to have a uniform material for all experiments.

EXPERIMENTAL.

A modified Ringer's fluid of the following composition behaves as serum of the same alkaline reserve in regard to pH, CO_2 tension, and total CO_2: 0.7 per cent NaCl, 0.04 per cent KCl, 0.02 per cent $CaCl_2$, 0.027 per cent $MgSO_4.7H_2O$, 0.2 per cent $NaHCO_3$, 0.001 M H_3PO_4. We have used this fluid in experiments on living animals with gratifying results. It should not remain open to the air, as it thus becomes too alkaline.

Our first problem was the determination of the alkaline reserve of the serum to be used in further work. We tried the compensation dialysis method of Michaelis and Rona, and found the alkaline reserve of ox serum apparently to vary from 0.03 to 0.04 N, but the osmotic pressure of the proteins and the varying permeability of the collodion membranes introduced errors, and our results may be too high. If serum is titrated with acid, using the hydrogen electrode as indicator, some means must be taken to remove the CO_2 or keep its tension constant in order to determine the correct end-point in the titration. If hydrogen is bubbled through the serum in an ordinary hydrogen electrode, some serum is carried away in the foam. In order to obviate this difficulty, we used the rotating hydrogen electrode previously used with bicarbonate solutions (McClendon, *b*). Volumetric flasks of the same size were filled with the serum and a different quantity of acid was added to each flask. The flasks were shaken and portions of the serum transferred successively from each flask to the rotating electrode, and a stream of the gas was passed through. The pH of the serum from each flask as well as the acid added was recorded, and these data were used to construct a titration curve from which the end-point in the titration could be

determined with accuracy (being the pH of distilled water at the same CO_2 tension). Owing to the time required for a single titration, we made no attempt to get the normal alkaline reserve of any species of animal by this means. The blood had been defibrinated in open dishes, and CO_2 allowed to escape. It is well known that the alkaline reserve of serum decreases when CO_2 escapes from the defibrinated blood, due to an ionic exchange with the corpuscles. The results reported apply to serum prepared as described.

Having determined the alkaline reserve of a series of sera, we added $NaHCO_3$ or acid to portions of serum to increase the range of alkaline reserve.

The experiments were made in a room kept at 20° by means of an electric apparatus. On a few days when the outside temperature rose above 20° the room was cooled by means of ice placed in front of an electric fan. A highly sensitive galvanometer was used with the potentiometer.

About 1.5 cc. of serum was placed in the rotating electrode, and a mixture of H_2 and CO_2 passed through it from the gas mixer. The stop-cocks were closed and the pH was determined, then the serum was used for the determination of the total CO_2 in the Van Slyke apparatus. In order to avoid some loss of CO_2 that always occurred when the serum was measured in a pipette, the serum was allowed to run quickly into the cup of the Van Slyke apparatus and a measured quantity allowed to run down into the graduated portion. The remainder of the serum was washed out of the cup with distilled water and the cup dried with filter paper. A correction was applied for the reversal in curvature of the meniscus and the volume of the hole in the stop-cock. 0.6 N HCl was introduced into the apparatus until the total fluid was 2.5 cc. This concentration of HCl has about the same absorption coefficient as serum for CO_2 (0.822 at 20°). The determination was that recommended by Van Slyke. The CO_2 pumped out was absorbed by NaOH and the CO_2 remaining in the acidified serum was calculated in each determination, then the total CO_2 was reduced to 0° and 760 mm.

The results of the experiments are given in Figs. 1 and 2. From Fig. 1 the pH may be determined from the CO_2 tension and alkaline reserve, or the CO_2 tension may be determined from the

pH and alkaline reserve, or the alkaline reserve may be determined from the pH and CO_2 tension.

The alkaline reserve may be approximately determined from the alveolar CO_2 tension alone, since the pH of blood in the arteries is remarkably constant, being about 7.33–7.5 (the variation being due largely to the method of determination). If the serum is allowed to come to equilibrium with the alveolar air at 20° the

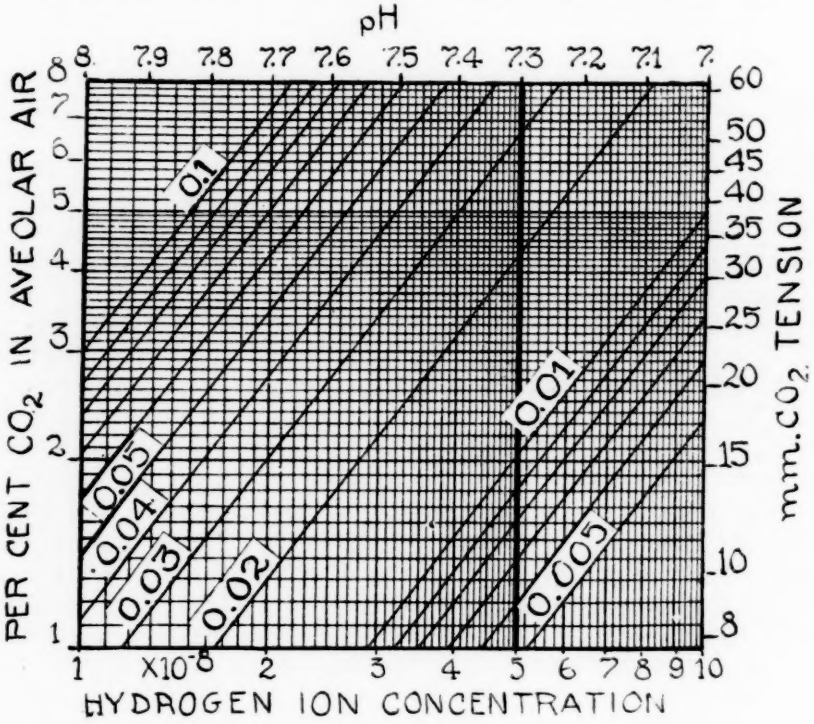

FIG. 1

pH is about 0.15 lower and in our experiments was 7.3; hence the alkaline reserve is represented by that diagonal passing through the intersection of the alveolar CO_2 abscissa with the pH 7.3 ordinate. In other words the alkaline reserve is nearly half the alveolar CO_2 tension in atmospheres, and can be determined more accurately than this from Fig. 1. In order to bring the blood or serum at 20° to the same pH as in the arteries, its CO_2 tension

must be lowered about 25 per cent. If the blood is drawn from the artery directly into a tube that may be closed so as to prevent

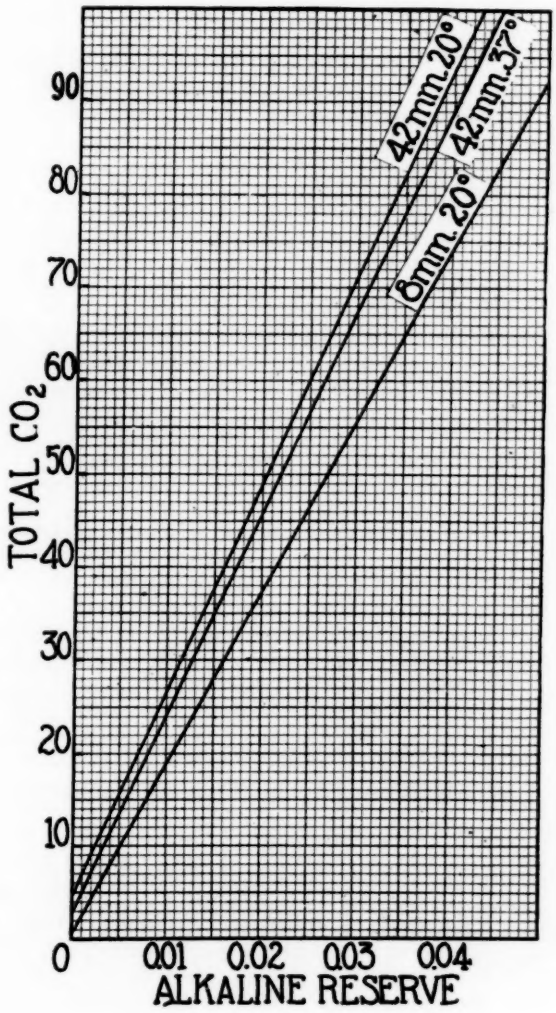

FIG. 2

gain or loss of CO_2 and cooled to 20°, the pH remains the same but the CO_2 tension falls about 25 per cent. Fig. 1 would represent the relations at 37° if the numbering of the pH scale at the

top were increased by about 0.15, or so that the heavy ordinate is numbered about pH 7.45 instead of 7.3. That is to say, the numerical relations between alkaline reserve and CO_2 tension remain the same, and it seems unnecessary to draw another figure for 37°. The rise of 0.15 pH due to rise in temperature from 20° to 30° at constant CO_2 tension is uncertain. We obtained values from 0.11 to 0.17 on serum, blood, and bicarbonate solutions. For a given difference of temperature the rise is less the higher the initial temperature.

Because war conditions have separated the authors from each other and from some of their notes, it is impossible to give the results in detail, but it seems worth while to report at present the mean of the results obtained.

From Fig. 2 the alkaline reserve may be determined from the total CO_2 at a given CO_2 tension and temperature, but a slight error in tension or temperature has little effect on the total CO_2. (The effect of change in temperature was calculated from a large number of experiments on bicarbonate solutions.) The data may be condensed into the following empirical formulas:

20°, 42 mm. CO_2 tension, total CO_2 = 4.5 + 2,180 × alkaline reserve.
20°, 8 " " " " " = 0.87 + 1,820 × " "
37°, 42 " " " " " = 2.9 + 2,155 × " "

If arterial blood is drawn into a tube in such a way that the serum may be collected without loss of CO_2, the alkaline reserve may be calculated from the total CO_2, by using the formula:

pH 7.5, total CO_2 = 2,250 × alkaline reserve.

If serum is subjected to alveolar CO_2 tension at 20° the alkaline reserve may be calculated from the formula:

pH 7.3, total CO_2 = 2,333 × alkaline reserve.

If the CO_2 is removed from serum until the pH rises to 8, the alkaline reserve may be calculated from the formula:

pH 8, total CO_2 = 1,850 × alkaline reserve.

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Van Slyke, Cullen, and Stillman, *Proc. Soc. Exp. Biol. and Med.*, 1914–15, xii, 184.
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Since this paper was written the attention of the senior author has been called to a paper by Hasselbalch (*Biochem. Z.*, 1916, lxxviii, 112). Hasselbalch's observation that rise of temperature does not change pH if CO_2 does not escape was previously observed by McClendon and Magoon (and is also true of bicarbonate solutions). Hasselbalch's observation that rise in temperature at constant CO_2 tension increases the pH of bicarbonate solutions is also true of blood. His observation that corpuscles behave as buffers was previously published by McClendon and Magoon and is explained perhaps by the ionic exchange between corpuscles and plasma. Obviously the pH of the corpuscle interior is not measured by the hydrogen electrode, but a change in pH of corpuscle may cause a change in pH of plasma or serum.

THE EFFECT OF TEMPERATURE ON THE REACTION OF LYSINE WITH NITROUS ACID.*

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(Received for publication, July 12, 1917.)

Lysine, unlike the rest of the amino-acids with exposed amino groups, requires a considerably longer period to react completely with nitrous acid. This has been demonstrated by Van Slyke in two papers.[1] The reason for this is explained by the possible slower reactivity of the ϵ-amino group. In the first paper it was reported that the reaction is not complete until 30 minutes, but the second paper would indicate that the reaction goes to completion in 15 minutes. In the Van Slyke method of protein analysis it is, therefore, recommended to shake the hexone bases for half an hour, so as to be absolutely sure of the completion of the reaction of lysine.

It was incidentally noted by Dr. Roxas, working in this laboratory last summer, that at high temperatures lysine reacts with both amino groups in 5 minutes. This observation invited further research upon the influence of temperature on the reaction of lysine with nitrous acid.

In order to demonstrate clearly the meaning of our data, Van Slyke's results on the behavior of lysine towards nitrous acid are quoted below.

* Published with the permission of the Director of the Wisconsin Agricultural Experiment Station.

[1] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 199; 1912, xii, 282.

TABLE I.

*Reaction of Lysine with Nitrous Acid (Van Slyke).**
(For Each Analysis 0.0888 Gm. of Lysine Picrate Was Used.)

Time of reaction.	N gas.	Temperature.	Pressure.	Amino N.	Calculated.
min.	*cc.*	*°C.*	*mm.*	*per cent*	*per cent*
5	9.90	19	758	6.36	7.47
15	10.75	19	758	6.87	7.47
15	11.20	20	758	7.06	7.47
30	11.50	19	758	7.39	7.47
30	11.80	20	758	7.54	7.47
50	11.70	19	758	7.51	7.47

* Van Slyke, *J. Biol. Chem.*, 1911, ix, 199.

TABLE II.

*Reaction of Lysine with Nitrous Acid (Van Slyke).**

Time.	Weight.	N gas.	Temperature.	Pressure.	Amino N.	Calculated.
min.	*gm.*	*cc.*	*°C.*	*mm.*	*per cent*	*per cent*
5	0.2	25.4	24	764	7.13	7.47
15	0.2	26.7	24	764	7.49	7.47
30	0.2	26.7	24	764	7.49	7.47

* Van Slyke, *J. Biol. Chem.*, 1912, xii, 282.

The interesting fact in connection with Van Slyke's work indicated above is that at 19°C., 6.36 per cent amino or 84.01 per cent of the total amino nitrogen reacted in 5 minutes, while at the higher tenperature, 24°C., 7.13 per cent amino or 95.2 per cent of the total amino nitrogen reacted in the same period of time.

EXPERIMENTAL.

Lysine picrate was prepared according to the Kossel and Kutscher method, which on analysis proved to be pure. Roxas' observation was corroborated by working with lysine picrate in an incubator room at 37°C. It will be noted from the following table that *both amino groups reacted in 5 minutes.* In view of this consideration, it appeared important to determine the lowest temperature at which *both* amino groups would react in 5 minutes,

the idea being to reduce the time of shaking the hexone bases when working at high temperatures.

Due to the practical inconvenience of handling the Van Slyke apparatus in a thermostat, the use of the latter as a means of controlling temperature was not attempted. The temperatures lower than 37°C. were obtained by the use of an electrical hot plate placed a short distance from the apparatus. This work was conducted in a small room and the temperatures desired were easily controlled within 1°. The data are recorded in Table III.

TABLE III.

Effect of High Temperatures on the Reaction of Lysine with Nitrous Acid.

Weight.	Time.	Temperature.	Pressure.	N gas.	Weight.	Amino N.	Amino N, per cent of total.
gm.	*min.*	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*	
0.04000	5	37	741	5.95	3.0035	7.51	99.60
0.04000	5	37	741	5.95	3.0035	7.51	99.60
0.03636	5	29.5	746	4.92	2.6019	7.16	94.96
0.03636	5	32	746	5.25	2.7483	7.55	100.10
0.03636	10	30	746	5.28	2.7438	7.54	100.00

It will be noted that 32°C. is the lowest temperature for the completion of the reaction in 5 minutes; also that 30°C. is the lowest temperature for the completion of the reaction in 10 minutes.

Attention was next directed to the effect of low temperatures on the reaction of lysine with nitrous acid. To secure these results advantage was taken of the winter months, and after several trials, constant low temperatures within 1–2° were obtained by open windows. Fully 10 minutes at the constant low temperature were allowed to elapse before shaking was started. It was found impossible to work below 1°C. on account of the appearance of ice in the burette of the apparatus. The data are given in Table IV.

It will be observed that low temperatures have a peculiar retarding effect on the reaction of lysine with nitrous acid; also that at 0–1°C. only 50 per cent of the total amino nitrogen reacted. It seems that at this low temperature and at the low concentration employed (0.36 to 0.40 per cent) only one-half, or probably

TABLE IV.

Effect of Low Temperatures on the Reaction of Lysine with Nitrous Acid in 5 Minutes.

Weight.	Temperature.	Pressure.	N gas.	Weight.	Amino N.	Amino N, per cent of total.
gm.	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*	
0.03636	5–6	748.5	3.15	1.8881	5.19	68.83
0.03636	4–5	748.5	3.00	1.8057	4.97	65.94
0.03636	3–4	748.5	2.85	1.7225	4.74	62.86
0.03636	0–1	748.5	2.25	1.3767	3.78	50.10
0.03636	0–1	748.5	2.25	1.3767	3.78	50.10
0.03636	0– −1	748.5	2.15	1.3209	3.63	48.14

the α-amino group reacted, and that the ε-amino group was inactive.

Since, according to Van Slyke and Birchard,[2] it is the exposed ε-amino group of lysine in the protein molecule which makes the protein directly reactive to nitrous acid, it was of interest to investigate the effect of low temperatures on the reaction of several proteins with nitrous acid. Casein,[3] dissolved in dilute sodium carbonate, and gliadin[4] (in more concentrated solution), dissolved in dilute sodium hydroxide, were employed, as outlined by Van Slyke and Birchard. Although an appreciable volume of gas was obtained at room temperatures in 5 minutes, almost a negligible volume of gas (within the limits of experimental error) was obtained in the cold. Zein, which is reported as containing no lysine, gave a very small volume of gas at room temperature.

This phase of the work was conducted in a refrigerator room and very satisfactory conditions for controlling temperature were afforded. It will be noted that the ε-amino group, the only reacting group in this case, is considerably retarded, almost excluded, at the low temperatures.

In the process of the retardation of the reaction of lysine with nitrous acid at the low temperatures employed, there is a possi-

[2] Van Slyke, D. D., and Birchard, F. J., *J. Biol. Chem.*, 1913–14, xvi, 539–547.

[3] 1 gm. of casein was dissolved in 100 cc., and 2 cc. portions were used in the micro apparatus.

[4] 5 gm. of gliadin were dissolved in 100 cc., and 10 cc. portions were used in the large apparatus.

TABLE V.

Effect of Temperature on the Reaction of Proteins with Nitrous Acid in 5 Minutes.

Protein.	Weight.	Temperature.	Pressure.	N gas.	Amino N.
	gm.	*°C.*	*mm.*	*cc.*	*mg.*
Zein	0.02	25	740.5	0.06	0.00272
"	0.02	25	740.5	0.06	0.00272
Casein	0.02	27	740.5	0.63	0.33667
"	0.02	27	740.5	0.64	0.34201
"	0.02	3	748.0	0.05	0.03020
"	0.02	5	748.0	0.21	0.12580
Gliadin	1.00	24	746.0	1.10	0.60225
"	1.00	25	746.0	1.20	0.65700
"	1.00	0–1	748.0	0.05	0.03069
"	1.00	0–1	748.0	0.05	0.03069

bility of the additional retardation due to the α-amino group of the lysine molecule. In order to determine whether low temperatures have a retarding effect on the α-group of amino-acids, the effect of low temperatures on the reaction of alanine with nitrous acid was tried. The results are given in Table VI.

TABLE VI.

Effect of Temperature on the Reaction of Alanine with Nitrous Acid.

Weight.	Temperature.	Pressure.	N gas.	Weight.	Amino N.	Calculated.
gm.	*°C.*	*mm.*	*cc.*	*mg.*	*per cent*	
0.012	23.5	743	3.48	1.90216	15.90	15.73
0.012	23.5	743	3.48	1.90216	15.90	15.73
0.012	3.0	745	3.28	1.97170	16.47	15.73
0.012	3.0	743	3.28	1.91250	15.91	15.73

It will be noted that low temperatures have no retarding effect on the reactivity of the α-amino group of alanine. It is, therefore, safe to conclude from the preceding results that *low temperatures have a retarding effect on the ε-amino and not on the α-amino group of lysine.*

SUMMARY.

1. At definite concentrations, 32°C. is the lowest temperature at which both amino groups of lysine react with nitrous acid in 5 minutes.

2. At certain definite concentrations, 30°C. is the lowest temperature at which both amino groups of lysine react with nitrous acid in 10 minutes.

3. It is suggested that at temperatures of 30°C. and above, 10 or a maximum of 15 minutes would be more than sufficient for shaking the hexone bases in the Van Slyke method of protein analysis, at any concentration, instead of 30 as was the practice heretofore.

4. At temperatures of 1°C. and slightly below and at definite concentrations it is possible to render the ϵ-amino group of lysine entirely inactive.

METHODS FOR THE DETERMINATION OF BLOOD SUGAR IN REFERENCE TO ITS CONDITION IN THE BLOOD.

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In a previous article von Hess and McGuigan (1) studied the condition of the sugar in the blood and concluded that it is all free and exists in the blood as in a water solution. They realized that anesthesia may change the blood sugar in amount and probably in condition, and to control this as far as possible they used various anesthetics, but always obtained the same results. While everything points to the sugar being free there still remains the possibility of a loose combination of sugar, similar to that of oxygen with hemoglobin, which might be disrupted by such mild manipulations as they used in their dialysis work. The present work was undertaken to determine whether or not we could find any basis for such an assumption. At some stages of the work it seemed we had evidence of such a combination, but closer investigation gives a more satisfactory explanation and confirms the previous conclusion, that the blood sugar is free in solution. There are some differences, however, between the reactions of dextrose in a water solution and dextrose in a protein-free blood filtrate. It is this difference we think which is mainly responsible for the great variations reported in the literature regarding the normal level of the sugar in the blood.

In the present work we have used both the Bertrand (2) and the Lewis-Benedict (3) methods. Under some conditions there is a great discrepancy in the results of the two methods and an examination of this difference we think throws light on the cause of the reported variations in the normal level of the blood sugar. While engaged in this work we noticed the article by W. B. Smith

(4) showing that picric acid does not interfere with the reaction of Fehling solution. If such be the case it renders the Bertrand and Benedict methods directly comparable since the conditions of precipitation can be made exactly alike. The differences, however, between the analyses of the blood picric acid filtrate by the two methods were so enormous that we doubted the truth of the statement. However, we found in control work, with dextrose in water, that picric acid does not interfere with the Bertrand method, as the following results show: A solution of dextrose was prepared, approximately 0.1 per cent *in water* and determined by the Bertrand method with the results: normal, 0.110, 0.110 per cent. After the addition of 1 gm. of picric, an amount greatly in excess of saturation but which went into solution on boiling, the results were: 0.113, 0.115 per cent. This slight increase may be considered negligible and within the limits of error. It is constant, however, and due to a salt action, as salts to some extent increase the reduction of alkaline copper, as the results of Bertrand show.

The influence of salt on the result of the Bertrand method is shown by the following figures:

Sugar in water.	Sugar in salt.			
I.	II. 2 per cent Na_2SO_4	III. 5 per cent Na_2SO_4	IV. 12 per cent Na_2SO_4	V. 25 per cent Na_2SO_4
per cent	*per cent*	*per cent*	*per cent*	*per cent*
0.084	0.085	0.090	0.110	0.110
0.085	0.090	0.094	0.110	
0.085			0.110	
0.085			0.110	
0.085			0.110	

Beyond 12 per cent the influence of the salt does not change. We quote this salt, and 10 to 20 per cent, because hitherto in the precipitation of the proteins from blood, we have used sodium sulfate and acetic acid to a great extent. The influence of the acetic acid will be emphasized later. The most probable explanation of the salt action is its influence on the boiling point of the solution.

Having convinced ourselves that picric acid does not interfere with the results of the Bertrand method, and what slight influence it has is to increase the yield of sugar, we were prepared to investigate the differences in the results of the Bertrand and the Lewis-Benedict methods. The comparison here is very direct since we use picric acid to remove the proteins in each case.

Methods.

To obtain blood, dogs were bled from the jugular vein with a hypodermic syringe. The blood was usually oxalated, sometimes defibrinated, at other times used directly without oxalate after quickly measuring before clotting could occur. 10 cc. samples were taken for each separate determination. This amount was for convenience only, as 5 cc. are sufficient. The blood was laked by adding it directly to 60 cc. of water. After 15 minutes laking 1 gm. of solid picric acid was added and thoroughly mixed with the blood solution until saturation. The volume was then made to 150 cc. with saturated picric acid in water, filtered, and 100 cc. of the filtrate (6.66 cc. of blood) were used for the analyses. This makes a convenient volume to handle, it filters rapidly, and is sufficiently large to minimize slight changes due to evaporation on boiling. The picric solution was brought to boiling, and to it were added 60 cc. of mixed Fehling solution (also boiling), and the boiling was continued for 2 minutes, timed with a sand glass, then filtered through asbestos, washed free from picric acid with hot water, and the whole transferred to a beaker with about 40 to 50 cc. of hot water. To this were added 25 cc. of 5 per cent ferric sulfate in 20 per cent sulfuric acid, and this solution was titrated with permanganate, while still hot. Each specimen should be titrated at about the same temperature and under the same conditions.

The potassium permanganate solution used was standardized by preparing a water solution of dextrose of 0.1 per cent, and 10 cc. of this were placed in 90 cc. of water, or if using salt to precipitate proteins, in the same strength of salt as used in the experiment, and the permanganate was brought to such a strength that 1 cc. represents 0.001 gm. of glucose.

The Lewis-Benedict method was carried out as recommended by Meyers and Bailey (5). The following table shows some of the results we have obtained by these methods directly, and also by Bertrand's method after "hydrolysis."

Any method of removal of the proteins of the blood, where acid is avoided, will give results similar to these quoted. We had corroborated the work by the use of (1) alcohol, (2) methyl alcohol, (3) colloidal iron, (4) sodium sulfate, (5) basic lead acetate, (6) dialysis of the serum.

Bertrand, direct.	Lewis-Benedict.	Bertrand. after "hydrolysis," or directly with 5 per cent KOH, Fehling solution.
0.010	0.100	0.067
0.010	0.080	0.090
0.030	0.119	0.110
0.054	0.114	0.116
0.058	0.102	0.096
0.030	0.095	0.092
0.035	0.100	0.100
0.023		0.085
0.015	0.080	0.075
0.030		0.123
0.020	0.094	0.100
0.020	0.075	0.090
0.010	0.078	0.075
0.022	0.114	0.117
0.017	0.090	0.196
0.015		0.120
0.018	0.080	0.112
0.018		0.130
Average.....0.0241	0.0944	0.0991
Comparable average.....0.015	0.0944	0.0954

In seeking for the cause of the great difference between the Bertrand and the Benedict methods we naturally suspected the presence of a sugar combination—"virtual sugar"—or a sugar of the maltose type, and so hydrolyzed and obtained a rise in the amount, and a figure which in most cases agrees very closely with the Benedict method. There were many facts, however, which made us doubt that there was an actual hydrolysis. First, to get the highest results, we had to use a certain amount of acid which reduced the alkalinity from 12.5 per cent KOH to 5 per cent, and second, the time necessary for the completion of the supposed hydrolysis was very short, and higher results were obtained if the acid used was not neutralized before applying the Fehling solution. Finally we found if the acid were added directly to the Fehling solution, *i.e.*, lessened alkalinity of the Fehling, the same results were obtained as after the supposed hydrolysis. It is evident, therefore, that lessened acidity and not hydrolysis is

the true explanation. The reason for not neutralizing after hydrolysis and before using the Fehling was due to the fact that there is great variability in the alkaline strength of Fehling's solution as recommended in standard works, and even without neutralizing we had still a strongly alkaline liquid that corresponded to many so called Fehling solutions. Controls with this solution tested in water solution gave good results. The results in Table III were obtained by adding 4 cc. of acetic acid to the 100 cc. of picric acid filtrate of the blood solution used for the analysis.

The alkalinity of Fehling's solution has been given (6) as 125 gm. of KOH in 500 cc. Mathews' (7) and many other texts adopt this amount. The United States Pharmacopeia VII used the same amount but in Edition VIII reduced this to 75 gm., and in the most recent edition, IX, reduces this to 50 gm. in 500 cc. This makes the alkalinity of the mixed fluids 5 per cent KOH in the last case against 12.5 per cent in the earlier editions. Claude Bernard at first used about 11.5 per cent NaOH and later about 6 per cent of the mixed fluids. Fehling's original solution (8) was about 5.6 per cent NaOH, which he afterward reduced to about 4.75 per cent NaOH. At the present time we may say the alkalinity of the complete Fehling's solutions as recommended varies from about 5 per cent KOH or less to 12.5 per cent NaOH. It is because of this great variation that we were in the beginning somewhat careless in neutralizing the acid added for hydrolysis and we were justified in this because a water solution dextrose yield is but slightly changed even by great variations in the alkalinity of the Fehling. *In a protein-free blood filtrate, however, the condition is vastly different.* Benedict's and Bertrand's methods with dextrose in water solution agree exactly, but note the difference when they are applied for the determination of the sugar in the blood.

In the preceding cases and throughout, except as mentioned, we used a Fehling solution of the following composition:

I.		II.	
$CuSO_4$	34.65	Rochelle salt	173
Water to	1,000 cc.	NaOH	125
		Water to	1,000 cc.

Except for the substitution of NaOH for KOH, this solution has the composition of that recommended by Bulletin 107 since they advise dilution with an equal quantity of water before use. It is also of the same composition so far as the alkali goes as recommended by Hammarsten (9).

With a water solution of dextrose almost theoretical results can be obtained with this solution and Bertrand's method as we have used it. The above results are so striking and so out of harmony with current opinion that errors in method, or the presence of interfering bodies, must be seriously considered. Traces of protein or other bodies which hold cuprous oxide in solution were thought of. The presence of such bodies seems improbable, as is shown by the following work.

First, dextrose added to normal or to diabetic blood can be recovered. This has been shown in many cases. Second, the result of dialysis shows that whatever the nature of such a body may be, it is dialyzable. Dialysis does not preclude peptone bodies which perhaps would be the protein that would interfere most. Such a body, however, would seem to be excluded by the lessening of the alkalinity which removes the effect and which could not remove the interfering body. While the presence of peptone thus seems improbable, its possibility still remains, for we know that traces of peptone beyond detection or recognition markedly influence the Bertrand method, while they have little influence on Benedict's method. If such a body be present, however, its solvent effect is limited because added sugar can be recovered and changes in the blood sugar are easily recognized. Third, the action of ether. In anesthetized animals, and in diabetics we can detect no change in the blood protein. Here again, however, the presence of a disturbing body might well escape us because the amount of sugar in these cases is so large that a change which would make a large percentage change in normal blood would be relatively insignificant here.

Instead of a protein the interfering body might be a dextrin-like body, but the fact that it will dialyze and is so easily "hydrolyzed" removes the probability of a dextrin.

Quite recently Scott (10) has suggested the presence of a lecithin combination, but this seems improbable since extraction of the picric filtrate or of the blood with ether before precipitation does not change the sugar in any way. This, however, does disprove Scott's contention, as he was working under different conditions. It is also not a glycogen-like body because when alcohol is used to precipitate the proteins, the glycogen would be removed, and yet we obtain results *similar* to the picric acid method.

The only investigator, so far as we know, who has reported the normal level of the blood sugar nearly as low as the figures we give for the direct determination after precipitation by picric acid is Shaffer (11). In the early part of the work we were much influenced by Shaffer's results and statements, and thought that our picric acid method showed that most of the sugar was in a combined form. Further work, however, has convinced us that this is erroneous, that the previous work of von Hess and McGuigan is correct, and that Shaffer also was unaware of the influence of highly concentrated alkaline Fehling's solutions on protein-free blood filtrates. Shaffer used the 12.5 per cent KOH Fehling.

Effect of Ether Anesthesia on Blood Sugar.

Dog No.	Condition.	With and without ether anesthesia.	Sugar, using KOH.	
			12.5 per cent.	5.0 per cent.
			per cent	*per cent*
1	Eck fistula 2 months old	None.	0.027	0.100
	" " 2 " "	3 hrs.	0.132	0.237
2	Reversed Eck fistula 6 weeks old	None.	0.020	0.090
	" " " 6 " "	3 hrs.	0.090	0.180
3	Eck fistula, healed	None.	0.040	0.160
	" " "	3 hrs.	0.180	0.210
4	Normal	None.	0.056	0.078
	"	10 min.	0.115	0.133
5	"	None.	0.066	0.102
	"	10 min.	0.073	0.140
6	"	None.	0.020	0.110
	"	30 min.	0.040	0.140
	"	120 "	0.080	0.156
7	"	None.	0.052	0.104
	"	3 hrs.	0.205	0.260
8	"	3 "	0.125	0.163
9	"	3 "	0.090	0.125
10	"	3 "	0.142	0.210
11	"	3 "	0.075	0.108

His average for the normal blood sugar in four dogs was 0.036, 0.020, 0.046, and 0.026 per cent, figures that agree closely with ours by the direct picric acid method. His results after anesthesia show a considerable rise, showing also, we think, agreement with our explanation that added sugar by whatever cause can be recovered. The low results he at first obtained we think are due to the solvent action of the strong alkali on the cuprous oxide which is increased by an unknown body in the blood. This solvent action is fully satisfied by the amount of sugar in the normal blood so that added sugar or the increase due to anesthesia may be recovered. This solvent action is much greater in a blood filtrate than in a water solution.

The influence of ether is thus seen to increase the amount of sugar when determined by the Bertrand method after precipitation of the proteins by picric acid. This action is not due to the breaking up of a sugar combination because blood extracted directly with the ether is not changed, nor is the picric blood filtrate changed by similar treatment. The experiment also shows that Eck fistula does not seem to change the action of ether on the blood sugar.

Morphine also increases the sugar as determined by this method, as is shown by the following experiment.

		12.5 per cent KOH.	5 per cent KOH.
Normal dog.	Blood sugar direct.	0.015 per cent	0.08 per cent
3 hrs. after 0.15 gm. of morphine.		0.165 " "	0.225 " "

The opinion has been expressed by more than one writer that the sugar in the blood exists in combination with something of the nature of an amboceptor. There are some facts brought out by the picric acid precipitation of proteins that might be used to sustain this theory. Since picric acid shows that the blood sugar is not a simple solution of glucose in water, its use also permits the testing of the amboceptor hypothesis and to a considerable extent supports that theory.

For example, Shaffer found that dextrose added to a solution of sugar that had fermented with yeast could not be completely recovered. We have been able to confirm this as the following experiment will show.

2 gm. of cane sugar were dissolved in 500 cc. of tap water, one-half of a cake of yeast was added, and the whole was placed in an incubator at 38°C. for 48 hours. After this time:

1. Direct test with Fehling solution showed no dextrose present.
2. 100 cc. boiled with 4 cc. of acetic acid and Fehling solution showed without neutralization a trace reduction = 0.012 per cent.
3. 10 cc. of dextrose = 0.12 per cent dextrose added and 78 per cent of it recovered.
4. The same boiled with 4 cc. of acetic acid = 100 per cent recovered.

Sugar added to a yeast fermented residue, however, can be recovered or lost, depending on the alkalinity of the Fehling solution used.

One-half of a cake of yeast was added to 500 cc. of 0.2 per cent dextrose solution and the mixture allowed to stand for 1 week in an incubator at 40°C. A known amount—0.1 per cent—of dextrose was then added. When determined with Fehling's solution of the strength given by the Pharmacopeia, which is 5 per cent KOH, this showed in water solution 0.105 per cent; with 12.5 per cent NaOH, Fehling, 0.034 per cent. Increasing the copper in Fehling solution has the same influence as decreasing the alkali. In the preceding case when 12 per cent NaOH was used and 7 per cent $CuSO_4$ instead of 3.5 per cent, we obtained 0.09 per cent; 20 per cent NaOH and 7 per cent $CuSO_4$, 0.1 per cent.

This variation in the copper content of Fehling's solution is better shown in the following experiment.

A dog was bled under ether and the blood defibrinated and precipitated with picric in the usual way. The alkaline tartrate part of the Fehling is constant, 125 gm. of KOH and 346 Rochelle Salt in one liter, but the copper solution varies as indicated.

2	per	cent	Cu,	0.021	per	cent	dextrose	in	the	blood.
4	"	"	"	0.044	"	"	"	"	"	"
8	"	"	"	0.096	"	"	"	"	"	"
12	"	"	"	0.157	"	"	"	"	"	"

At this last strength of copper the black oxide is precipitated so that the last figure is not reliable.

With the same strength of Fehling's solution as tested against a known dextrose solution in water the variation is much less, *e.g.*, a 0.102 per cent dextrose solution gives:

2	per cent	Cu,	0.07	per	cent.
4	"	"	0.188	"	"
8	"	"	0.102	"	"
12	"	"	0.102	"	"

Since the utilization of the dextrose by the yeast cell is probably by the same mechanism as in the animal cell, this masking or combining with the sugar suggests a similar preliminary step in the animal body. Accordingly we have tested blood by:

1. Adding sugar directly to it when fresh, and determining the amount of the added dextrose that could be recovered.
2. The same addition after blood had undergone glycolysis.
3. Normal and diabetic blood has been compared.

In the normal blood the *added* sugar can be recovered, while in blood that has been freed from sugar by glycolysis added sugar is immediately masked as in yeast fermentation and cannot be recovered until after the addition of acid. This suggests that the blood sugar normally is in combination, and that when the sugar is removed by glycolysis, the amboceptor (12) still retains the power to unite with free dextrose converting it into the same form as the dextrose normally existing in the blood.

In like manner we have determined the sugar in normal blood directly by the picric acid Bertrand method, and have found that all the added sugar can be recovered, while in blood that has undergone glycolysis a certain amount combined with the amboceptor and can be recovered only after adding acid or reducing the alkalinity. The following experiments illustrate this.

Experiment No.	Normal sugar. By using Fehling 12.5 per cent NaOH.	After adding acid.	Sugar added in:	Recovered.
	per cent			
I	0.01		0.102 per cent water.	0.124
	0.01		0.102 " " "	0.125
II	0.02		0.102 " " picric.	0.142
III	0.023	0.085	0.098	0.100
IV	0.017	0.123	0.102	0.158

The recovery of more sugar than is added is, we think, due in part to the liberation of acid radicles when Cu_2O is formed. Thus H_2SO_4 then acts as so much added acid and sensitizes the Fehling solution, which then reacts with some of the potential sugar; and

in part it is due to the fact that weaker alkali in the Fehling normally gives a higher result.

After Glycolysis.

Experiment No.	Time of glycolysis.	Sugar added.	Recovered.
	hrs.		
I	48	0.102	0.046
	48	0.102	0.060
II	18	0.102	0.075

In normal blood by the picric acid method the average blood sugar direct is about 0.01 per cent. In sensitizing the Fehling by the addition of acid this amount rises to about 0.10 per cent. If dextrose be added to the normal filtrate containing 0.01 per cent we recover more than is added and this is probably because the reduction of the Cu causes a liberation of acid which sensitizes Fehling's solution by reduction of the alkalinity.

Normal blood was glycolyzed until it showed no sugar when tested with the Fehling solution. The Benedict method showed 0.016; Fehling containing 2.5 per cent NaOH or 10 per cent Na_2CO_3 gave 0.01 per cent. If now to 100 cc. of this glycolyzed blood picric acid filtrate we add 10 cc. of dextrose solution—or 0.102 per cent—and determine how much of this can be recovered by varying the alkalinity of Fehling's solution, we find for the original undiluted Fehling—12.5 per cent NaOH—only 60 per cent recovered, or 0.060. 5 per cent NaOH gives 0.110; 2.5 per cent gives 0.120 per cent. We thus find that added sugar can be recovered if not too much alkali is added or if sodium carbonate be used instead of KOH.

Controls with water solution gave with 12.5 per cent NaOH 0.102 per cent; with 5 per cent NaOH, 0.110 per cent. With the weaker alkali consequently we recover the total sugar, added and actual.

The following results show how changing the alkalinity influences the yield of the sugar as determined by Fehling's solution—Bertrand.

NaOH	Dog's blood in Fehling.
per cent	*per cent*
12.5	0.018
5	0.090
4	0.105
2.5	0.122

The Lewis-Benedict method on this blood gave 0.106 per cent.

Increasing the amount of copper in the Fehling has the same effect as decreasing the alkalinity, as the following analysis of dog's blood will show.

$CuSO_4$ *per cent*	12.5 per cent NaOH. *per cent*
2	0.021
4	0.044
8	0.096
12	0.157

In the last case some black oxide is precipitated.

A solution of dextrose in water or in picric acid solution is not nearly so much influenced by this variation of the copper, or by changes in the alkalinity.

$CuSO_4$ *per cent*	12.5 per cent NaOH. *per cent*
2	0.070
4	0.088
8	0.102
12	0.102

The Difference between Normal and Diabetic Blood.

We have examined the blood of several normal and diabetic persons which, at one stage of the work, we thought showed a significant difference. When examined more closely we think no such difference exists. The ratio of the amount of sugar after hydrolysis, or that determined by the 5 per cent KOH Fehling is striking.

Ratio in Normal Dogs.

Direct.	After hydrolysis or by 5 per cent KOH Fehling.	Ratio.
0.048	0.140	1:3
0.010	0.067	1:7
0.028	0.070	1:2.5
0.025	0.092	1:4
0.020	0.110	1:5
Average of 12		1:4

The influence of anesthesia, as is well known, raises the blood sugar, and if continued long enough may cause a glycosuria. It also changes this ratio remarkably.

I. Normal	0.020	0.110	1:5
After 2 hours ether anesthesia	0.08	0.156	1:2
II. 10 min. ether	0.052	0.104	1:2
III. After 3 hrs. ether	0.205	0.260	1:1.2

Ether always reduces this ratio, likewise anything that raises the amount of sugar in the blood. Averaging six dogs under ether for 1 hour, the ratio fell from 1:4 to 1:1.3. In a case of adrenalin glycosuria without ether the ratio was practically 1:1. In one case of phlorhizin diabetes, in which, as is well known, the blood sugar does not increase, the ratio did not change.

In normal human beings the ratio is practically the same as in normal dogs, as the following table will show.

No.	Indirect.	Hydrolyzed.	Ratio.
I	0.025	0.090 *	1:3.6
II	0.035	0.160	1:4.8
III	0.082	0.230	1:3
IV	0.082	0.240	1:3

Human diabetic blood again shows almost a ratio of 1:1 as the following will show.

No.	Indirect.	Hydrolyzed.	Ratio.
I	0.172	0.256	1:1.5
II	0.290	0.307	1:1.05
III	0.670	0.730	1:1.1

Some of this diabetic blood was allowed to glycolyze in the incubator at 40°C. until the sugar had all disappeared, and then a known amount of glucose was added. Of the added glucose in two cases only 40 and 60 per cent was recovered when analyzed by the strong alkali Fehling. So that if there be such a thing as an amboceptor, it acts, so far as we can make out, as strongly in diabetic blood as in the normal.

If we analyze the cases above cited it will be seen that, while the ratio differs enormously, the actual difference between the sugar obtained by the direct picric acid precipitation and after hydroly-

sis is about the same in all cases. For instance in the worst diabetic the difference between 0.730 and 0.670 is 0.06, and this is approximately the figure in most of the cases where the ratio is 1:4 or 1:5. Whatever the cause may be it acts in all cases but is more striking in those cases with an initial low figure. Since normally strong alkali to some extent dissolves cuprous oxide, in blood filtrates this solvent action is much greater than in water solution. The cause is not known. It is, however, organic, not inorganic, because blood ash added to sugar has no such influence.

SUMMARY AND CONCLUSIONS.

An investigation of the blood sugar was undertaken with special reference to its condition in the blood and to the difference between normal and diabetic blood. It was found that picric acid does not interfere with the use of Fehling solution or its modifications. This allows a direct comparison of the two most used methods, the Lewis-Benedict and the Bertrand. By the use of picric acid to remove proteins and the subsequent use of the Bertrand method it was found that when a Fehling solution which contains 12.5 per cent KOH is used very low sugar yields were obtained. In some cases only about one-tenth of that was obtained by the Benedict method. When 5 per cent KOH Fehling was used results agreeing closely with the Benedict method were obtained. The difference is not due to the picric acid directly because picric acid in water solution has no influence and other methods of removing the protein give similar results. Alcohol, colloidal iron, and sodium sulfate all give considerably lower results with the higher alkaline Fehling. The picric acid, however, is the most pronounced. Solutions of dextrose in water are somewhat lower with the stronger alkali Fehling but this difference is relatively small in comparison with the differences in the blood filtrate. The cause of the difference has not been definitely decided but apparently it is something of an organic nature which is not removed by the picric acid. Perhaps it is in the nature of, or at least acts in a manner similar to, creatine or peptone. It is not because the stronger alkali destroys sugar under these conditions because after boiling with the stronger alkali Fehling the higher yield of sugar can be obtained by the addition of acid suf-

ficient to reduce the alkali from 12.5 per cent to 5 per cent KOH. Also with added sugar, or by increase in the sugar content of the blood as in diabetes, or in anesthesia, or in adrenalin glycosuria and similar conditions, the whole increase can be obtained, which would not be the case if the alkali were destroying the sugar. This recovery of the increased sugar greatly changes the ratio obtained by the use of the two concentrations of alkali or between the results of the stronger alkali and the result of the Benedict method. This ratio roughly changes from 1:4 to 1:1. If this ratio has any significance it relates to the amount of interfering substance in the blood under the different conditions. Whatever this body is, it exerts very little influence on the Benedict method and this method seems to be the one of choice for blood sugar determination. The Bertrand method carried out as we have used it also gives good results but the strength of the alkali in the Fehling solution is important and should not be over 5 per cent KOH. We think the varying strength of the alkali used is the most important cause for the great variation in the normal level of the blood sugar reported in the literature. This level for man and dogs as originally reported by Bernard is about 0.1 per cent. The present work corroborates the work of von Hess and McGuigan, showing that the sugar in the blood reacts as it does in a water solution.

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THE ORIGIN OF CREATINE. II.*

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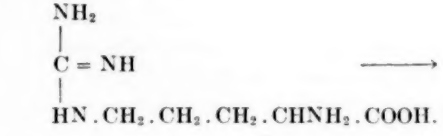
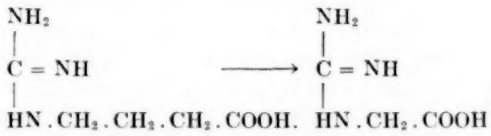
If arginine is partly converted into creatine by the animal tissues, glycocyamine may be regarded an intermediate product in this reaction.





Therefore it is important to determine whether the animal organism possesses the power to convert glycocyamine into creatine. Considerable work has been done on this problem but the question has not been conclusively decided. Czernicki,[1] using the zinc chloride precipitation method, obtained an increase of creatinine in the urine of rabbits after feeding glycocyamine but not when glycocyamidine was fed. This author does not regard his work as decisive. Jaffé[2] also determined the sum of creatine and creatinine in the urine and muscle of rabbits after feeding and injecting glycocyamine. He also employed the Neubauer zinc chloride method. This author found that glycocyamine, when

* The experimental data are taken from a dissertation submitted by Harry M. Hines as a partial requirement for the degree of Master of Science, State University of Iowa, Iowa City.

[1] Czernicki, W., *Z. physiol. Chem.*, 1905, xliv, 294.

[2] Jaffé, M., *Z. physiol. Chem.*, 1906, xlviii, 430.

injected or fed, led to an increase in the creatine and creatinine fraction both in muscle and urine. Dorner,[3] a pupil of Jaffé, repeated the latter's experiments but employed the more accurate colorimetric method. He found an increased excretion of creatine after the administration of glycocyamine, but also found that muscle *in vitro* possessed the power to methylate glycocyamine. Mellanby[4] criticizes Jaffé's technique and Dorner's experiments. He finds no consistent increase in the creatine content of chicken muscle after the administration of glycocyamine, but regards his work as inconclusive. More recently Palladin and Wallenburger[5] have apparently obtained relatively large increases in creatine when rabbit's muscle was permitted to act on glycocyamine at incubator temperature, and also when rabbits were injected with glycocyamine.

Our work on this problem may be divided into three parts. At first it was necessary to see whether creatine could be accurately determined in the presence of glycocyamine according to the conventional methods. We found that an acid solution sufficiently strong to convert creatine into creatinine would usually effect a partial conversion of glycocyamine into glycocyamidine and as the latter gives the picric acid reaction, the results for creatine were too high. Both Jaffé and Dorner separated creatine and creatinine from glycocyamine by exhaustive extraction with alcohol. We find that 95 per cent alcohol will extract sufficient quantities of glycocyamine to interfere with the creatine determinations. The solubility of glycocyamine in 95 per cent alcohol is 0.02 per cent, that is 20 mg. dissolve in 100 cc. of boiling 95 per cent alcohol.

The second part of our work was concerned with the action of freshly hashed muscle on glycocyamine *in vitro*. Under these conditions, an increase of creatine was never encountered.

Finally we resorted to injection, perfusion, and feeding experiments on rabbits, dogs, and men. While the results of these experiments were not uniform, we obtained, in some cases, an increased elimination of creatine after the injection of glycocyamine.

[3] Dorner, G., *Z. physiol. Chem.*, 1907, lii, 225.

[4] Mellanby, E., *J. Physiol.*, 1908, xxxvi, 447.

[5] Palladin, A., and Wallenburger, L., *Compt. rend. Soc. biol.*, 1915, lxxviii, 111. This reference was only obtainable in abstract form.

EXPERIMENTAL.

The glycocyamine was obtained through the interaction of guanidine and chloroacetic acid according to the procedure of Ramsay.[6] The yield was 65 per cent, based on the amount of chloroacetic acid used. The guanidine required for this reaction was synthesized from calcium cyanamide, according to the excellent method of Levene and Senior.[7]

Determination of Creatine in the Presence of Glycocyamine.

Palladin and Wallenburger determined the creatine in muscle by heating the extract on a water bath for 3 hours in an approximately 0.6 N hydrochloric solution as suggested by Riesser.[8]

Experiment A.—100 cc. of a 1 per cent solution of glycocyamine in approximately 0.6 N hydrochloric acid were heated on a boiling water bath for 3 hours, then diluted to 200 cc. To one of two flasks, each containing 5 mg. of creatinine, were added 5 cc. of the glycocyamine solution (25 mg. of glycocyamine), and the intensity of the color was compared, after the addition of picric acid and alkali. The color of the glycocyamine flask was deeper than the control. This difference was equivalent to 0.8 mg. of creatinine for 25 mg. of glycocyamine or 3.2 for 100 mg.

Experiment B.—To two of four flasks, each containing a known quantity of creatine dissolved in 275 cc. of approximately 0.6 N sulfuric acid were added 100 mg. of glycocyamine. After heating on the boiling steam bath for 3 hours, the intensity of the Jaffé reaction was determined by means of the colorimeter and expressed as creatinine.

With	glycocyamine	38.97 and 39.36 mg.,	mean	39.16
Without	"	35.8 " 35.9 "	"	35.85

Difference, for 100 mg. of glycocyamine, 3.31 mg.

Experiment C.—A similar experiment in which 0.66 N sulfuric acid was used, showed an increase corresponding to 3.9 mg. of creatinine per 100 mg. of glycocyamine.

Experiment D.—A fourth experiment in which diluted muscle extract and 0.66 N sulfuric acid was used showed that 100 mg. of glycocyamine increased the color proportionately to 4 mg. of creatinine.

Solubility of Glycocyamine in Boiling 95 per Cent Alcohol.—An excess of analytically pure glycocyamine was boiled with 95 per cent alcohol under reflux for 1 hour, then filtered through a hot steam jacketed funnel into a tared flask of known volume.

[6] Ramsay, H., *Ber. chem. Ges.*, 1908, xli, 4385.

[7] Levene, P. A., and Senior, J. K., *J. Biol. Chem.*, 1916, xxv, 623.

[8] Riesser, O., *Z. physiol. Chem.*, 1913, lxxxvi, 415.

After evaporation of the alcohol on the steam bath, the flask was dried to constant weight and weighed. It was found that 57 cc. of boiling 95 per cent of alcohol dissolved 0.0115 gm. of glycocyamine or 100 cc. 0.020 gm.

Weyl's Reaction.—Both Jaffé and Dorner placed considerable importance on Weyl's test as a means of determining glycocyamidine in the presence of creatinine. We were unable to obtain sharp reactions in the concentrations with which we were dealing, so we preferred to control our experiments as described below.

Experiments in Vitro.—Freshly hashed rabbit muscle or dog liver was weighed into a flask containing glycocyamine dissolved

Weight of muscle.	Time at 38°C.	Glycocyamine added.	Creatine.	Weight of muscle.	Time at 38°C.	Glycocyamine added.*	Creatine.
gm.	*hrs.*	*mg.*	*per cent*	*gm.*	*hrs.*	*mg.*	*per cent*
Experiment E.				Control.			
7.293	24	40	0.577	7.529	24	40	0.546
7.724	24	40	0.561	8.672	24	40	0.564
7.996	48	33	0.596	7.714	48	40	0.596
6.426	48	40	0.595	7.061	48	40	0.600
7.337	48	40	0.563				
Average			0.578	Average			0.576
Experiment F.				Control.			
7.146	24	50	0.461	7.081	24	50	0.470
6.784	46	50	0.470	6.093	46	50	0.471
Average			0.465	Average			0.470
Experiment G.				Control.			
Weight of liver.				Weight of liver.			
6.370	36	50	0.0567	6.129	36	50	0.0587
9.039	42	50	0.0444	8.384	42	50	0.0520
7.147	63	50	0.058	8.105	106	50	0.056
Average			0.053	Average			0.056

* Glycocyamine added at the time of the determination.

in isotonic Henderson's phosphate solution. After the addition of toluene, the flask was placed in the incubator for from 24 to 96 hours and the creatine content determined according to the method of Janney and Blatherwick.[9] The absence of bacterial growth was confirmed by culture. The controls which had remained in the incubator were analyzed after the addition of an equivalent amount of glycocyamine. In this way the color due to glycocyamine was accounted for. As is evident from the tables, our results do not indicate methylation of glycocyamine *in vitro* by muscle or liver.

Injection and Feeding Experiments.

Rabbits were placed in a metabolism cage on a diet of carrots. The urine was gathered in 24 hour periods and diluted to a defi-

Experiment H.—A gray rabbit weighing 2,540 gm. was used for this experiment. At the conclusion of the experiment, the muscle contained 0.452 per cent of creatine, 72.55 per cent of water, and 3.71 per cent of nitrogen.

Experiment H.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
2	84	2	0	12	97	0	120
3	120	6	0	13	100	11	400
4	93	10	0	14	99	39	350
5	77	2	0	15	102	44	350
6	110	2	0	16	108	54	350
7	62	22	0	17	93	62	350
8	83	9	0	18	102	61	100
9	86	1	0	19	99	54	200
10	112	1	0	20	100	51	0
11	91	0	0	21	112	40	0
				22	106	15	0
Average..	91.8	5.5		Average.	101.6	39.2	
				23	107	2	0
				24	106	4	0

* Creatine is expressed as creatinine.

[9] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.

nite volume. During the second period, they were injected with known amounts of glycocyamine subcutaneously. Creatine was determined by heating the urine on the water bath with 0.66 N sulfuric acid for 3 hours.

A quantity of glycocyamine greater than that which could be present in the urine of the injected animals was added to normal rabbit urine and its effect on the creatine estimation determined. It was found that this quantity of glycocyamine (400 mg.) increased the daily creatine from 10 to 12 mg.

If we allow 12 mg. for the effect of dissolved glycocyamine in the urine and 6 mg. for the average daily excretion during the preliminary period, we still have an excess of more than 21 mg. to be ascribed to the influence of glycocyamine.

Experiment I.—A female pregnant rabbit[10] was placed in a cage on a diet of carrots. At the beginning of the experiment it weighed 2,435, at the end 2,564 gm. The creatine content of its muscle at the conclusion of the experiment was 0.448, the water content 74.22, and the nitrogen 3.58 per cent.

Experiment I.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	60	6	0	10	112	4	300
2	106	5	0	11	92	5	150
3	88	11	0	12	76	37	300
4	84	3	0	13	116	39	300
5	84	7	0	14	71	35	300
6	124	12	0	15	111	30	300
7	71	1	0	16	74	17	0
8	111	2	0				
9	72	0	0				
Average...89		5		Average.. 93		24	
				17	103	0	0

* Creatine expressed as creatinine.

[10] The pregnant condition of this animal was determined when the experiment was in progress.

If we allow 12 mg. for the glycocyamine effect and 5 mg. for the daily average excretion during the preliminary period, 7 mg. remain which we are inclined to ascribe to the effect of the glycocyamine.

We are unable to account for the low concentration of creatine in the muscle tissue.

Experiment J.—The subject of this experiment was a male rabbit weighing 2,174 gm. at the beginning and 2,155 gm. at the end of the experiment. The diet consisted of carrots.

Experiment J.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	74	4	0	9	105	10	150
2	78	0	0	10	68	7	300
3	57	4	0	11	96	4	150
4	50	6	0	12	85	25	300
5	53	1	0	13	Lost.		300
6	100	8	0	14	71	31	300
7	63	3	0	15	87	16	300
8	116	1	0	16	103	19	0
				17	53	25	0
Average...	74	3.4		Average..	83	17	
				18	90	0	0

* Creatine is expressed as creatinine.

After making the necessary deductions it is found that the excretion of creatine is increased during the second period by 1.6 mg. per day.

Experiment K.—The subject of this experiment was a bitch weighing 12 kilos. She was placed in a metabolism cage and fed on a mixture of skimmed milk powder, bread, lard, and agar. The experiment was started after the dog had been on this diet for 4 months. The urine was collected in 24 hour periods and made to a definite volume with the wash water from the cage. At the close of the experiment the dog weighed 15 kilos. 17 days later a small piece of the vastus externus muscle was removed from a right hind leg under anesthesia. It contained 0.35 per cent creatine,

73.71 per cent water, and 3.68 per cent nitrogen. 2 months later the corresponding muscle from the opposite side contained 0.318 per cent of creatine, 73.39 per cent of water, and 3.52 per cent of nitrogen.[11]

Experiment K.

Day.	Creatinine.	Creatine.*	Glycocyamine injected.	Day.	Creatinine.	Creatine.*	Glycocyamine injected.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	386	1	0	10	362	14	400
2	370	15	0	11	345	64	600
3	382	21	0	12	396	61	830
4	380	23	0	13	398	60	800
5	378	12	0	14	380	90	800
6	345	3	0	15	365	122	800
7	393	29	0				
8	364	14	0				
9	364	14					
Average	373	14.5		Average	374	68.5	

* Creatine is expressed as creatinine.

In previous experiments we found that 100 mg. of glycocyamine when heated with 0.66 N acid for 3 hours reduces alkaline picric acid proportionately to 3 mg. of creatinine. Assuming that all of the glycocyamine which was injected appeared in the urine we would deduct 24 mg. for the glycocyamine effect and 14.5 mg. for the daily average creatine elimination during the preliminary period. This leaves a daily average elimination of 30 mg. to be ascribed to the effect of glycocyamine injection.

Experiment L.—A man weighing 62 kilos, aged 23 years, and in perfect health, was placed on a practically creatine-free diet consisting of bread, milk, cereals, vegetables, and fruit. The urine was carefully collected in 24 hour periods. Creatine was determined by heating the urine on the water bath for 3 hours with N sulfuric acid. Glycocyamine was administered *per os*.

[11] In previous unpublished experiments we have found that the creatine content of dog muscle is markedly diminished after the animals have been kept in cages on the milk and bread diet for several months.

Experiment L.

Day.	Creatinine.	Creatine.*	Glycocyamine fed.	Day.	Creatinine.	Creatine.*	Glycocyamine fed.
	mg.	*mg.*	*mg.*		*mg.*	*mg.*	*mg.*
1	1,397	26	None.	10	1,358	81	1,000
2	1,376	15	"	11	1,362	24	2,000
3	1,378	23	"	12	1,342	33	2,000
4	1,398	27	"	13	1,282	83	2,000
5	1,320	15	"	14	1,342	53	3,000
6	1,214	0	"				
7	1,386	13	"				
8	1,367	57	"				
9	1,256	0	"				
Average	1,343	19.5		Average	1,337	55	

* Creatine is expressed as creatinine.

As considerable glycocyamine was undoubtedly present in the urine, the increased excretion of creatine during the second period is more apparent than real and is probably due to the presence of the guanidine acetic acid.

Experiment M.—A brown bull bitch weighing 12.3 kilos was fed on a diet consisting of skimmed milk powder, dry bread, lard, and agar, for 4 months prior to the operation. On March 7 a piece of muscle was removed from the right hind leg under aseptic precautions, and ether anesthesia. On March 14 the animal was given 200 mg. of glycocyamine twice a day and this was repeated for the next 8 days. On March 28 a corresponding piece of muscle was removed from the opposite side. The specimens of muscle were examined for creatine, water, and nitrogen.

Experiment M.

	Creatine.	Creatine per gm. of dry muscle	Water.	Nitrogen.
	per cent	*mg.*	*per cent*	*per cent*
Before glycocyamine	0.30	9.2	67.31	3.72
After "	0.31	11.0	71.84	3.44

We are unable to account for the difference in water content in the two samples of muscle, as the weight of the dog remained almost constant throughout the experiment. The increase of creatine is not convincing as we have no means of saying how much glycocyamine has been stored in the muscle tissue.

Experiment N.—A small dog, 9 months of age, weighing 4.5 kilos, was decerebrated under anesthesia and a piece of the vastus externus muscle was removed from one hind leg. Approximately 2 gm. of glycocyamine dissolved in isotonic acid phosphate solution were injected into the jugular vein. 5½ hours later a similar piece of muscle was removed from the opposite side. The creatine and water content of the muscle before the injection were 0.305 and 72.46 per cent, respectively, and after the injection, 0.316 and 71.47 per cent, respectively. In terms of dry substance, the creatine content of both samples is identical.

Experiment O.—The right hind leg of a dog weighing 15 kilos was perfused for 1 hour with 1.7 gm. of glycocyamine dissolved in 270 cc. of perfusion fluid. This consisted of 1.7 gm. of glycocyamine dissolved in 100 cc. of water, containing 3.28 cc. of 5 N hydrochloric acid. This was rapidly neutralized with an equivalent amount of sodium hydroxide solution and added to 150 cc. of the dog's own defibrinated blood. The pressure of the perfusate during the experiment fluctuated between 80 and 100 mm. of mercury. Only 110 cc. of fluid were recovered. The hamstring muscles were removed from both hind legs at the close of the experiment, and creatine and water determined. Creatine was determined according to the method of Janney and Blatherwick.[9] The perfusion technique has already been described.[12] Owing to its slight solubility in water, glycocyamine is difficult to perfuse in concentrations greater than that employed in this experiment.

Experiment N.

	Creatine.	Creatine per gm. of dry muscle.	Water.
	per cent	*mg.*	*per cent*
Perfused muscle	0.382	14.8	74.2
Unperfused "	0.374	13.4	72.12

The muscle of the perfused limb weighed exactly 1 kilo. If we assume that all of the glycocyamine contained in the perfusate was retained by the muscle tissue then 25.8 gm. of dry muscle tissue should contain 170 mg. of glycocyamine in addition to the creatine. If we deduct the chromogenic equivalent of this amount of glycocyamine,[13] which is 11 mg., from the apparent

[12] Baumann, L., and Marker, J., *J. Biol. Chem.*, 1915, xxii, 49.

[13] Janney and Blatherwick use 1 N acid for the conversion of creatine into creatinine. When 100 mg. of glycocyamine are heated with this concentration of acid for 3 hours, and alkaline picric acid is added to the neutralized solution a color develops which is equivalent to 5.5 mg. of creatinine.

creatine value for the perfused muscle, we find that 25.8 gm. of dry muscle contain 371 mg. of creatine or 14.4 mg. per gm. This increase is worthy of note. We hope to have opportunity to repeat this experiment.

CONCLUSIONS.

1. Our experiments do not offer any evidence for the methylation of glycocyamine by muscle or liver tissue *in vitro*.

2. The injection of glycocyamine into rabbits and dogs may be followed by an increased excretion of creatine.

THE PRODUCTION OF CREATINURIA IN NORMAL ADULTS.

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(Received for publication, July 28, 1917.)

As a result of numerous investigations made in the thirteen years which have elapsed since the publication of Folin's method for the determination of creatine and creatinine, it is now a generally accepted fact that creatine does not occur in the urine of normal men living on a creatine-free diet. Until a few years ago it was believed that this statement applied also to normal non-pregnant women.

In 1911 it was however pointed out by Krause[1] that the urine of normal women frequently contains small amounts of creatine. This creatinuria is, according to this investigator, associated with the sexual cycle, is always present after menstruation, and while in some individuals it may disappear during the intermenstrual period in others it persists.

In work recently published from this laboratory,[2] it has been shown that in children it is possible to increase or decrease the amount of creatine excreted in the urine by increasing or decreasing the quantity of protein (creatine-free) in the food. As a result of these experiments the suggestion was made that creatinuria in normal children is probably due to the high level of their protein intake. If this theory be true, it would seem possible that if the protein intake could be increased to a sufficiently high level, creatinuria in normal adults might also be obtained.

In order to test out this hypothesis we have carried our experiments on four normal adults, two men and two women, along the same general lines and by the same methods used in investigating this subject in connection with children.

[1] Krause, R. A., *Quart. J. Exp. Physiol.*, 1911, iv, 293.

[2] Denis, W., and Kramer, J. G., *J. Biol. Chem.*, 1917, xxx, 189.

Our first experiments were carried out with two normal women who had previously acted on several occasions as subjects for metabolism studies. Just previous to becoming subjects for this investigation these women had, in connection with another problem, been living for about 8 days on a creatine-free diet of low protein content. The urines passed on the last day of this period were examined for creatine but with negative results. Both subjects were then placed on a creatine-free diet containing the largest amount of protein they could take.

The diets used, which were the same for both subjects, were as follows:

High Protein Diets.

	1	2	3
Eggs	12	6	12
Gelatin, *gm.*	50		50
Cheese (neufchatel), *gm.*	200	50	200
Milk, *cc.*	500	500	200
Bread, *gm.*	300	400	
Sugar, "	50	50	
Butter, "	50	50	
Lemons			2
Orange			1
Tomato and lettuce salad			One portion (not weighed).

Low Protein Diet.

Bread, *gm.* 50	Baked apple, *gm.* 200	Cream, 40 per cent, *cc.* 200
Corn meal, *gm.* 75	Apple and celery salad, *gm.* 200	1 orange.
Potato, *gm.* 250	Butter, *gm.* 50	1 banana.
Lactose, *gm.* 100	Bacon fat, *gm.* 100	1 lemon.

The low protein diet was eaten by Subject I in the form given above; in the case of Subject II the corn meal was replaced by 200 gm. of potato.

From the results presented in Table I it will be seen that it was possible in the case of these two women to induce creatinuria by high protein feeding. After 5 days on the high protein Diet 1 Subject I was allowed to return for 3 days to the food which she ordinarily consumes, which consists largely of bread, vegetables,

and fruit, with a small amount of meat (approximately 50 gm.) once a day. Even this diet was apparently sufficiently low in protein to cause an immediate disappearance of all creatine

TABLE I.

Date.	I. Normal female, 38 years old, weight 91.0 kg.				II. Normal female, 22 years old, weight 53.6 kg.			
	Total nitrogen.	Preformed creatinine.	Creatine.	Diet.	Total nitrogen.	Preformed creatinine.	Creatine.	Diet.
May	gm.	gm.	gm.		gm.	gm.	gm.	
14–15					5.02	1.12	0	
15–16	5.8	1.11	0		8.46	1.12	0	1
16–17	12.6	—	—	1	16.87	1.13	0	1
17–18	19.76	1.31	0.12	1	24.58	1.25	0.13	1
18–19	16.27	1.20	0.09	1	20.06	1.22	0.37	1
19–20	20.35	1.33	0.16	1	24.43	1.21	0.35	1
20–21	20.00	1.46	0.21	1	19.60	1.19	0.22	1
21–22	12.28	1.21	0		12.66	1.28	0.24	Mixed.*
22–23	7.98	1.13	0	Mixed.*	18.45	1.12	0.18	1
23–24	9.80	1.13	0		21.88	1.16	0.13	1
24–25	11.79	1.21	0	2	20.59	1.18	0.15	1
25–26	15.41	1.23	0	2	21.28	1.21	0.18	1
26–27	12.25	1.18	0.10	2	22.65	1.18	0.17	1
27–28	11.94	1.10	0.12	2	—	—	—	1
28–29	14.97	1.19	0.13	2	17.22	1.05	0.14	Low.
29–30	8.25	1.14	0.10	Low.	7.59	1.10	0.10	"
30–31	6.66	1.21	0	"	6.58	1.10	0.10	"
31–June 1	6.25	1.17	0	"	7.75	1.08	0.09	"
June								
1–2	5.48	1.11	0	"	4.65	1.00	0	"
2–3	5.90	1.11	0	"	5.76	1.07	0	"
3–4	5.98	1.12	0	"	5.88	1.16	0	"
4–5	14.61	1.38	0.08	3	12.95	1.19	0.09	3
5–6	18.69	1.42	0.15	3	17.26	1.13	0.28	3
6–7	21.08	1.26	0.20	3	23.85	1.09	0.34	3
7–8	20.50	1.28	0.22	3	22.22	1.33	0.20	3

* Ordinary mixed diet with meat, once a day.

from the urine. An attempt was then made by feeding the high protein Diet 2 to obtain an idea as to the general level at which creatinuria could be induced but even on this moderate protein intake creatine again appeared.

A low protein diet was then consumed for 5 days with the resultant disappearance of all creatine after the 2nd day, and this was in turn followed by a 5 day period on a diet containing approximately the same amount of protein as that contained in the high protein Diet 1, but differing from Diet 1 in that it contained only an extremely small amount (probably not more than 2 or 3 gm.) of carbohydrate.

By this experiment it was believed that it might be possible to obtain evidence as to the effect, if any, produced by the ingestion of carbohydrate on creatine excretion. As will be seen creatine appeared on the 2nd day as was the case in the period in which the high protein Diet 1 (which contained 300 gm. of bread) was taken and remained at about the same level as that reached during the first period.[3]

An attempt was made to follow this period of high protein and low carbohydrate feeding by one containing the same amount of protein but with a much larger amount of carbohydrate than was given in the first period. This, however, proved to be impossible, as the large amount of carbohydrate consumed (300 gm. of lactose and 300 gm. of bread) produced in both subjects such marked digestive disturbances that they were compelled to abandon the experiment.

The experimental periods on Subject II were essentially similar to those just described for Subject I except that the high protein Diet 1 was continued for a much longer period than in the case of Subject I, and that no period of moderate protein feeding was introduced.

Having been successful in the production of creatinuria in women by forced protein feeding we next attempted to duplicate our results in men.

Our third subject was a man 25 years old, weighing 62.6 kg. For a period of 14 days he consumed the following ration daily.

Whole eggs	6	Bread, *gm.*	200
Egg whites	4	Macaroni, "	50
Gelatin, *gm.*	50	Potato, "	100
Cheese (American), *gm.*	100	Sugar, "	40
Milk, *cc.*	100	Butter, "	40

[3] During this period the urine was examined daily for acetone bodies by means of the Scott-Wilson reagent. In the case of Subject I a minute

During this period the urine was frequently examined for creatine but invariably with negative results. The mixed urines for the period were found to contain 251.1 gm. of nitrogen, or an average excretion of 18.0 gm. per day. An attempt to increase the protein intake was unsuccessful.

For our next experiment we used Subject IV, a man 20 years old, weighing 57.7 kg., who seemed possessed of a more capacious appetite than Subject III. For 12 days this man was kept on the same rations as had been used for Subject III. The mixed urines for the period were found to contain 227.9 gm. of nitrogen, or an average daily excretion of 19.1 gm.

Frequent attempts to find creatine in these urines were, however, invariably rewarded with negative results.

It was therefore decided to increase the protein intake, and for 5 days the ration consisted of:

Eggs	30	Chocolate, *gm.*	50
Milk, *cc.*	1,500	Bread "	150
Gelatin, *gm.*	50	Sugar "	50

The determination of the urinary nitrogen gave the following results for these 5 days: 22.9, 19.4, 32.7, 34.5, and 33.5 gm. In spite of the high level of nitrogen consumption no creatine could be detected in the urine at any time during the 5 days during which the experiment was continued.

In the present state of our knowledge of creatine metabolism the above results are difficult of interpretation. The hypothesis that the ease with which creatinuria can be induced in normal women may be due to their relative lack of muscular development (as compared with that of men) immediately presents itself, but the results recorded above cannot be interpreted as furnishing evidence in favor of such a theory.

SUMMARY.

In two normal women it was found possible to cause creatine excretion by feeding a high protein (creatine-free) diet, and to

trace of diacetic acid was found on the 3rd day and a larger amount (about 10 mg. calculated as acetone) on the 4th day. In the case of Subject II traces of acetone bodies appeared only on the 4th day.

cause the creatinuria so produced to disappear by the consumption of a low protein diet. In two men to whom a similar experimental procedure was applied we were unable to produce creatine excretion even when a sufficient amount of protein was consumed to cause the urinary nitrogen to rise to 34.5 gm. per day.

EXPERIMENTAL STUDIES ON GROWTH.

IX. THE INFLUENCE OF TETHELIN UPON THE EARLY GROWTH OF THE WHITE MOUSE.

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Object of the Experiments.

Previous investigations have shown[1] that the administration of tethelin, a lipoid extracted from the anterior lobe of the pituitary body,[2] leads to marked retardation of the growth of the white mouse during the first 10 weeks of the third or "adolescent" growth cycle, a retardation which is succeeded by pronounced acceleration. The effect of these phenomena is to completely distort the form of the curve of growth in the third growth cycle, giving the appearance of great prolongation and enlargement of the second growth cycle (which normally terminates at 5 weeks,[3]) and an acceleration and curtailment of the third growth cycle.

In the experiments referred to, the administration of tethelin was initiated when the animals were 5 weeks old and the second growth cycle had already begun to merge into the early stages of the third. The observed effects are therefore to be interpreted as effects of tethelin upon the third or final cycle of growth during which maturity of the sexual organs is attained, together with possible effects upon the residual growth still attainable by a continuation of the second growth cycle. It appeared of considerable importance to supplement these results by ascertaining the effects of tethelin upon the first (infantile) and second growth

[1] Robertson, R. B., *J. Biol. Chem.*, 1916, xxiv, 397. Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

[2] Robertson, *J. Biol. Chem.*, 1916, xxiv, 409; *Endocrinology*, 1917, i, 24.

[3] Robertson, *J. Biol. Chem.*, 1916, xxiv, 363.

cycles of the white mouse. The following experiments were accordingly undertaken.

Methods.

The method employed was similar to that utilized by Robertson and Cutler[4] in determining the effects of lecithin and cholesterol upon the growth of suckling mice. Since, during the first 3 weeks of extra-uterine development, mice are dependent upon the mother for their nutrition, administration of substances directly to the young by mouth is attended with difficulties, while the manipulations incident to hypodermic or intraperitoneal administration might very conceivably exert an effect upon the welfare and thus indirectly upon the growth of the animals which might mask or distort the effects which are the object of inquiry. We have therefore sought to influence the growth of the young during the period of lactation by administration of the tethelin by mouth to the mother, fully recognizing, however, that the interpretation of the results obtained during this period is complicated by the possibility that the quantity and quality of the mother's milk may be somewhat affected by the administration and also by the possibility that the tethelin may be utilized or destroyed by the tissues or in the mammary glands of the mother so that it may fail altogether to reach the young. The decided effects upon the growth of suckling mice obtained by Robertson and Cutler as a result of administering cholesterol to the mother, however, encouraged us to adopt this method in the present investigation.

During the period of growth subsequent to weaning (3rd to 5th weeks) and comprising the greater part of the second growth cycle the mother was removed from the cage containing the young which thereafter received the tethelin directly with their food. The administration of tethelin was discontinued at the end of the 5th week (*i.e.*, on the 35th day after birth) but the animals, thereafter fed upon a normal diet, were weighed on the 42nd and 49th days after birth in order to determine the residual or continued effects of the tethelin previously administered.

Sixty litters of mice were divided into three groups, A, B, and C. The division was made at the birth of the litters, the litters

[4] Robertson, T. B., and Cutler, E., *J. Biol. Chem.*, 1916, xxv, 663.

being alternated so that the first litter came in group A, the second in group B, the third in Group C, and so on. In this way the groups obtained consisted of nearly equal numbers of initially similar animals. The litters were kept in separate cages during the period of the experiment and the mother was supplied with an abundance of rolled barley and water and occasionally with fresh lettuce leaves and dried bread. In addition to this the control animals (Group A) each received daily 1 cc. of mixed yolk and white of egg, another group of animals (B) received the same amount of egg mixture to which, however, were added 10 mg. of tethelin dissolved in 0.2 cc. of distilled water, while the third group (C) received the same amount of egg mixture daily to which, after 14 days, *i.e.*, after the termination of the first or infantile growth cycle, were added 10 mg. of tethelin dissolved in 0.2 cc. of distilled water. The animals were weighed daily (with occasional omissions of 1 day) to the nearest cg., each mouse in all the litters being weighed separately. The litters were all kept in the same room and under identical conditions.

The dosage of tethelin was not modified with the growth of the animals. The dosage per gm. of body weight therefore diminished very rapidly with age. Excluding the uncertain proportion, very possibly, as we shall see, amounting to the whole of the substance administered, which may have been destroyed or appropriated by the tissues of the mother during the 21 days of lactation, the average dosages per gm. of the young amounted approximately to the following.

Age.	Approximate dosage per gm. of young.
days	*mg.*
0	1.33
7	0.67
14	0.52
21	0.40
28	0.31
35	0.26

This may be compared with the dosage, varying between 0.30 mg. at 5 weeks and 0.15 mg. at 1 year, administered to the mice employed in the experiments previously reported.

DISCUSSION.

The results obtained are shown in Table I and depicted graphically in Fig. 1, in which the continuous curve denotes the growth curve of the normal animals, the broken curve that of the animals which received tethelin from birth to 35 days, and the dotted curve that of the animals which received tethelin from 14 to 35

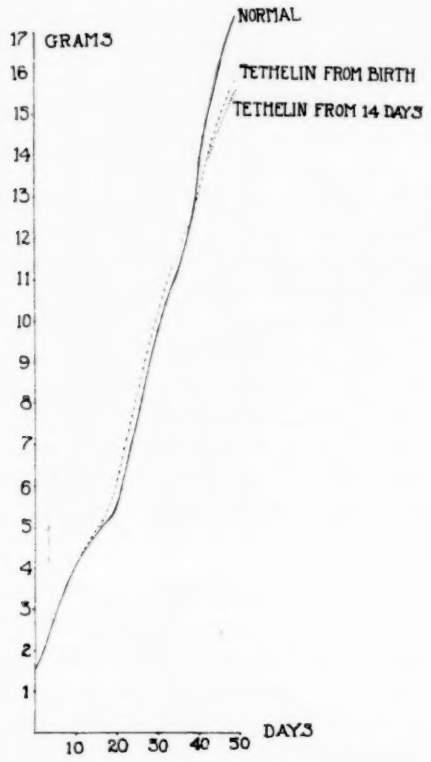

FIG. 1. The influence of tethelin upon the early growth of white mice.

days of age. It will be seen that prior to 14 days of age the administration of tethelin to the mother was absolutely devoid of effect upon the growth of the young, the two curves of growth being so nearly identical as to be indistinguishable from one an-

TABLE I.

Age.	A. Normal.		B. Tethelin.		C. Tethelin after 14th day.	
	No. weighed.	Average weight.	No. weighed.	Average weight.	No. weighed.	Average weight.
days		*gm.*		*gm.*		*gm.*
0	118	1.47	88	1.61	126	1.47
1	111	1.73	102	1.82	107	1.67
2	116	1.96	103	2.01	106	1.85
3	100	2.28	81	2.31	99	2.23
4	84	2.53	90	2.51	108	2.47
5	78	2.76	81	2.85	89	2.76
6	79	3.06	60	3.14	60	3.02
7	91	3.35	82	3.35	100	3.27
8	80	3.66	83	3.59	85	3.54
9	91	3.86	83	3.76	85	3.72
10	82	4.11	64	4.04	79	4.09
11	73	4.36	75	4.15	82	4.29
12	67	4.25	74	4.44	77	4.39
13	65	4.53	55	4.72	49	4.75
14	82	4.44	78	4.64	87	4.74
15	75	4.74	65	4.79	76	4.93
16	87	4.81	69	4.85	77	4.94
17	76	5.12	52	5.39	76	5.33
18	64	5.16	63	5.44	74	5.55
19	60	5.28	63	5.83	77	5.65
20	65	5.99	54	6.26	55	6.09
21	74	5.89	70	6.64	83	6.51
22	67	6.74	65	6.92	75	7.05
23	79	7.03	63	7.19	79	7.38
24	66	7.52	51	8.00	76	7.86
25	63	7.91	66	8.06	71	8.36
26	63	8.00	61	8.67	73	8.23
27	62	8.65	48	9.02	49	8.84
28	74	8.55	63	8.88	78	9.30
29	65	9.49	61	9.27	63	10.02
30	73	9.62	66	9.60	60	10.20
31	67	10.04	46	10.36	66	10.41
32	57	10.52	50	10.91	70	10.98
33	54	10.67	56	11.02	64	11.10
34	52	11.14	43	11.71	43	11.71
35	65	11.08	57	11.61	73	11.77
42	140	14.85	52	13.91	69	13.83
49	80	17.43	42	15.90	55	15.59

other in the diagram. After 14 days a noticeable acceleration of growth occurs, and that this acceleration is a genuine effect of the administration of tethelin is indicated by the fact that the growth curves of the two tethelin-fed groups overlie one another so as to be almost indistinguishable from one another in the diagram, while the normal (continuous) curve diverges from them as indicated in the figure. This acceleration persists until the culmination of the second and initiation of the third cycle after which a decisive retardation, as observed in the previously reported experiments, occurs. This retardation is exhibited notwithstanding the cessation of the administration of tethelin at 5 weeks, corresponding with the conclusion of the second cycle.

TABLE II.
Variability.

Ages.	A. Normal.	B. Tethelin.	C. Tethelin after 14th day.
days	*per cent*	*per cent*	*per cent*
0	18.4	17.7	21.2
7	20.8	17.5	21.8
14	20.3	20.6	22.2
21	28.7	20.2	27.1
28	26.2	26.1	25.0
35	26.2	23.2	22.7
42	20.5	21.6	20.1
49	16.0	18.5	16.8

The effects of the administration therefore persist for a considerable period after its discontinuance.

The variabilities of the weights of the three groups of animals at 7 day intervals computed in the manner described in previous communications,[5] are enumerated in Table II. No decisive effect of the administration upon the variability of the animals is observable, although the animals receiving tethelin display a slight tendency to diminished variability, the mean variability of the normal group during the period from and including 21 to 49 days being 23.5 per cent while the mean variabilities of the tethelin-fed groups during the same period were 21.9 and 22.3 per cent.

[5] Robertson, *J. Biol. Chem.*, 1916, xxiv, 363, 385, 397; *Am. J. Physiol.*, 1916, xli, 535.

No effect was observed of the administration of tethelin upon the date at which the eyes of the young opened, which is a very sharply defined criterion of development. Since the animals of Group C did not receive tethelin until the 14th day, the animals of both Groups A and C serve during the first 14 days of development as controls with which the development of Group B may be compared. It will be observed (Table III) that the average date at which the eyes opened in the young of group B is intermediate between the dates at which the eyes opened in the young of the two control groups. In view of the absence of effect upon growth in weight during the first 14 days, the lack of effect of administration of tethelin to the mother upon structural development as evidenced by the opening of the eyes was to be anticipated.

TABLE III.

Opening of Eyes.

Class.	Average age at which eyes opened.	Variability of age at which eyes opened.
	days	*per cent*
A (Normal)	14.4	7
B (Tethelin)	14.0	6
C (Tethelin after 14th day)	13.6	9
A + C	13.8	8

The comparative invariability of the period at which the opening of the eyes occurs has been commented upon elsewhere.[3,6] As will be observed on comparing the figures enumerated in Tables II and III the variability of the period at which the eyes open is correlated with the initial variability in weight of the young at birth, the least variable group in weight at birth displaying the least variable period at which the eyes open, while the most variable group in weight at birth (C) displays the most variable period at which the eyes open. Hence the attainment of this stage of development, although relatively so invariable a phenomenon, is nevertheless in some measure affected by the much more variable phenomenon of growth in weight.

[6] Daniel, J. F., *Am. Naturalist*, 1912, xlvi, 591.

SUMMARY.

From the total lack of effect of the administration of tethelin to the mother upon the growth of suckling young we may infer either that tethelin exerts no effect upon growth during the first (infantile) growth cycle or else, which is more probable, that the tethelin administered to the mother is not secreted as such to any appreciable extent by the mammary glands of the mother.

The administration of tethelin to the young subsequently to the 14th day, when their eyes are open and they have access to food other than that supplied to the mother, results in a noticeable acceleration of growth during the second growth cycle (2nd to 5th weeks), followed, upon initiation of the third cycle, by a marked retardation which evidences itself despite the fact that the administration of tethelin is discontinued at the end of the 5th week.

Variability of the period at which the eyes open is correlated with the initial variability in weight of the young at birth.

THE BLOOD LIPOIDS IN NEPHRITIS.

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At the time when bleeding was a common therapeutic practice nephritis was one of the conditions in which milkiness of the plasma was occasionally observed (1), and blood examinations in recent years give support to these earlier reports—indicating that there may be a disturbance of fat metabolism in this disease.

Thus Watjoff (2) found, in a case of nephritis, microscopically visible fat which stained with osmic acid. Bönniger (3) reported blood fat (total lipoids) high. Erben's (4) analyses showed increased values for fat and lecithin in a subchronic case. Greenwald (5) found high lipoid phosphorus in some of his series of nephritics. Chauffard, La Roche, and Grigaut (6) reported hypercholesterolemia in chronic nephritis with lipemia (milky plasma) in a case of uremia, and Widal, Weill, and Laudat (7) found lipemia frequently in nephritis. Henes (8) observed that the blood cholesterol was increased and that the increase was greatest in the most severe cases. He called attention, however, to the fact that in one fatal case with uremia the cholesterol value sank shortly before death. J. Müller (9) gave the following high values for the blood lipoids in a case of nephritic lipemia (blood taken post mortem): Total ether extract 3.6 per cent; neutral fat 2.15; cholesterol 0.84; lecithin 0.69. Schmidt (15) found in most patients with hypertension, in whom kidney function was not far from normal, that the cholesterol values were high, while if marked functional deficiency existed the values were normal or below. Epstein and Rothschild (10) found in chronic parenchymatous nephritis, particularly in the edematous stage, that the blood lipoids were very high—cholesterol up to 1.23 gm. per 100 cc. of blood, while in uremic cases, especially those with high nitrogen retention, the lipoids were much diminished—cholesterol as low as 0.08 gm. per 100 cc. The origin of the high lipoid they believed to be ingested or mobilized fat representing a condition of non-utilization, as evidenced by the fact that on a low fat diet the lipemia disappeared. Denis (11) found a notable increase in blood cholesterol in nephritis in only one case out of about fifty of various types examined. She suggests that the lack of high values in her series may have been due to a diet which contained little cholesterol.

While the published results are thus quite conflicting and while various factors such as the nature of the diet and the stage of the disease (in addition to alimentary lipemia, which obviously has not been taken into account in much of the work reported) appear to have an influence on the lipoid values, there is enough evidence to show that abnormalities in fat metabolism may be a feature of nephritis and that a further study of the blood lipoids in this condition is desirable. A series of blood samples from the severest cases of nephritis available at the time was therefore obtained from the Massachusetts General Hospital through the kindness of Dr. Denis. These samples were taken fasting (before breakfast) so as to exclude alimentary lipemia and to make the results comparable with those of normal individuals already reported, and were treated with alcohol-ether at the hospital as soon as obtained to obviate changes produced by standing.

Methods.

Certain changes have been introduced into the methods for determination of the blood lipoids since their first publication (12), which are briefly as follows.

Preparation of the Sample for Analysis.—When analyses are to be made of both whole blood and plasma about 10 to 12 cc. of blood are required. It is drawn from the vein into a syringe containing two drops of saturated sodium citrate, run out into a small flask, and well shaken. One drop of citrate for each 5 cc. has been found to prevent clotting if the blood is well shaken, and also to avoid hemolysis which may take place if more citrate is used. Oxalate has been found to produce hemolysis more frequently than citrate and therefore to be less desirable for human blood. From the well mixed sample in the flask, 3 cc. are measured off with a pipette and run slowly with stirring into 75 to 80 cc. of alcohol-ether in a 100 cc. graduated flask. The remainder of the blood is transferred to a graduated centrifuge tube (the graduation of these tubes is often poorly done and they should be calibrated before use) and centrifuged at 3,500 to 4,000 R. P. M. for 10 minutes. The level of the whole blood and of the corpuscle layer is then read off and the percentage of corpuscles calculated. If circumstances permit, the blood may be drawn

directly into a pipette by means of a needle and short piece of rubber tubing, and run from the pipette into the centrifuge tube, or if the lipoid values of the whole blood only are desired the blood may be run directly from the 3 cc. pipette into the alcohol-ether.

It is desirable to get the blood into the alcohol-ether as soon as possible after drawing so as to avoid possible changes in the lipoids by standing. After it is once precipitated it may be allowed to stand in the stoppered flasks for a week or more until a number of samples have collected or until it is convenient to proceed to the next step. Values do not appear to be affected in any way by this step. The filtered extracts when kept in tightly stoppered bottles in a cool place in the dark have been found to keep unchanged for at least 6 months so that the procedure offers a convenient way of collecting samples and storing them until it is convenient to make the analyses.

Total Fat.—In the determination of total fat the following changes have been made. To the blood extract after saponification are added 5 cc. of alcohol-ether, and the whole is raised to boiling. The liquid is then removed from the heat and after active bubbling has ceased the ether vapor is blown off, the beaker meanwhile being gently shaken. The 5 cc. of standard solution are heated in the same way (a few grains of coarse sand are added to promote even boiling). This treatment removes most of the ether, which if allowed to remain tends to produce bubbles in the water solution and also to cause differences in color (brownish tints) in one or other solution, due to slight differences in the aggregation of the precipitated material. Both standard and test solutions are thus treated throughout in as nearly the same way as possible so that the changes in volume produced by the loss of the ether are the same in each. To each of standard and test solutions are now added 50 cc. of distilled water, the solutions well stirred to ensure complete solution of the soaps, and the readings made according to the original directions. If the drying of the saponification mixture has been carried too far or if the temperature at the end of the drying has been too high, low values will be obtained, due probably either to baking of the mixture on the bottom of the beaker or to partial destruction of the cholesterol by the hot concentrated alkali. For this

Blood Lipoids in Nephritis.

	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Fat.		Total fatty acids / Lecithin			Lecithin / Cholesterol			Plasma total lipoids.	Kidney function.	Non-protein nitrogen.	Remarks.
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.				
	per cent																			*per cent*	*mg.*	
Normal average.		0.36	0.39	0.32	0.30	0.20	0.42	0.22	0.23	0.20	0.13	0.04	1.25	1.90	0.79	1.35	0.9	2.1	0.7			
1 S 220	45	0.42	0.33	0.53	0.32	0.18	0.50	0.16	0.14	0.18	0.12	0.18	1.30	1.83	1.06	2.0	1.3	2.8	0.50	60	43	
2 O'B 206	43	0.54	0.49	0.60	0.29	0.16	0.46	0.18	0.18	0.18	0.30	0.14	1.8	3.0	1.3	1.6	0.9	2.6	0.71	55	32	
3 S 207		0.38			0.14			0.15					2.7			0.9				40	30	
4 R 224		0.48			0.29			0.17					1.6			1.7				45	71	
5 J 200	35	0.39	0.45	0.30	0.26	0.14	0.48	0.19	0.17	0.23	0.29	0.0	1.5	3.2	0.6	1.4	0.8	2.1	0.67	30	40	Acute glomerular nephritis.
6 O 227	33	0.41	0.40	0.45	0.25	0.14	0.47	0.19	0.18	0.20	0.23	0.12	1.6	2.9	0.95	1.3	0.8	2.4	0.62	20	51	Dyspnea.
7 R 218	37	0.48	0.48	0.48	0.37	0.27	0.57	0.22	0.19	0.27	0.22	0.08	1.3	1.8	0.8	1.7	1.4	2.1	0.76	20	58	
8 B 201	34	0.46	0.52	0.34	0.26	0.15	0.47	0.25	0.22	0.30	0.32	0.0	1.8	3.5	0.7	1.0	0.7	1.6	0.76	20	107	
9 I 225	33	0.55	0.40	0.86	0.32	0.14	0.70	0.18	0.16	0.25	0.24	0.37	1.7	2.8	1.2	1.8	0.9	2.8	0.60	15	65	Dyspnea.
10 H 203	25	0.53	0.59	0.35	0.31	0.24	0.52	0.28	0.30	0.22	0.30	0.0	1.7	2.5	0.67	1.0	0.8	2.4	0.96	10	47	
11 K 202	19	0.57	0.56	0.60	0.27	0.18	0.65	0.19	0.18	0.23	0.34	0.14	2.1	3.1	0.9	1.4	1.0	2.8	0.77	10	107	Acute nephritis.
12 H 217	34	0.42	0.47	0.34	0.40	0.26	0.55	0.22	0.24	0.19	0.24	0.0	1.1	1.7	0.6	1.8	1.1	2.9	0.84	10	55	Uremia.
13 P 213	40	0.40	0.30	0.47	0.25	0.14	0.42	0.16	0.16	0.16	0.14	0.18	1.6	2.1	1.1	1.6	0.9	2.6	0.50			Acute nephritis, pneumonia.
14 S 212	20	0.64	0.66	0.56	0.21	0.19	0.29	0.20	0.18	0.28	0.46	0.35	3.0	3.3	1.9	1.1	1.0	1.0	0.90			

15 E 223		0.47			0.33			0.19					1.4			1.7				4	45	Acute nephritis, edema.
16 M 221	32	0.53	0.53	0.53	0.34	0.27	0.49	0.22	0.22	0.22	0.26	0.18	1.6	2.0	1.1	1.5	1.2	2.2	0.84	5	111	Very dyspneic.
17 " 222	24	0.63	0.67	0.50	0.36	0.24	0.74	0.21	0.21	0.21	0.42	0.0	1.8	2.8	0.7	1.7	1.1	3.5	0.95			Coma, death.
19 M 205	36	0.57	0.64	0.45	0.32	0.16	0.60	0.22	0.17	0.30	0.45	0.0	1.8	4.0	0.7	1.5	0.9	2.0	0.85	3	69	Marked acidosis, death.
20 G		0.60			0.26			0.21					2.3			1.2						Coma, death.
21 H 228	39	0.59	0.62	0.54	0.29	0.23	0.39	0.21	0.23	0.17	0.37	0.26	2.0	2.7	1.4	1.2	1.0	2.3	0.92		89+	Coma and death 1 week later.
22 O'F 219	14	0.49	0.47	0.61	0.30	0.25	0.60	0.16	0.15	0.17	0.23	0.19	1.6	1.9	1.0	1.9	1.7	3.5	0.69	0	232	Blood pressure 205, coma, and death.
23 C 204	13	0.63	0.63	0.63	0.25	0.18	0.70	0.19	0.18	0.25	0.42	0.13	2.5	3.5	0.9	1.3	1.0	2.8	0.85	0	187	Uremia, death, phosphates increased. Blood sugar 0.22.

* These values were obtained from the records of the Massachusetts General Hospital.

reason the drying should be done on a water bath rather than on an electric stove. The use of 50 cc. of water instead of the original 100 cc. gives a solution which is more easily read and when most of the ether is removed as above there is no trouble with bubbles.

Cholesterol.—Very few changes have been made in the method as described. A gentle air blast is used to hasten evaporation of the blood extract and to prevent over-heating, which produces a brownish tint and destroys part of the cholesterol. The directions for the production of the color have been slightly modified. Because of the high price of satisfactory acetic anhydride the amount used has been reduced from 2 cc. to 1 cc. for each determination. The smaller amount has been found entirely adequate for the amounts of cholesterol determined. The acetic anhydride obtainable at the present time is frequently of poor quality, either colored or developing a color in the determination. Distillation has been found to improve it. The temperature at which the color is produced is within a degree or so of 22°C., rather higher than lower. This temperature is the ordinary room temperature in most laboratories.

Lecithin.—The strychnine molybdate precipitation is now used altogether instead of the less convenient and less sensitive silver precipitation. The directions for the use of this reagent in the determination of lecithin have been given previously (13). A dilute sulfuric acid (one part of concentrated acid with three parts of water) is used for neutralization instead of the concentrated acid which was originally recommended because of the necessity with the silver precipitation of keeping the volume of solution small. A number of determinations indicate that, with the molybdate precipitation, the use of cane sugar solution and second heating recommended in the earlier directions is probably not necessary and it has been omitted in the later determinations.

The results of the determinations of the blood lipoids are given in Table I, the values being expressed in gm. per 100 cc. of blood.

The cases are arranged in the table in approximately the order of their severity and, for completeness, the values for non-protein nitrogen (in mg. per 100 cc. of blood) and kidney function (as determined by per cent excretion of phenolsulfophthalein), obtained from the records of the Massachusetts General Hospital, are included.

RESULTS AND DISCUSSION.

Total Fatty Acids.—High in both plasma and corpuscles.

Lecithin.—In the plasma lecithin was generally normal or below while in the corpuscles it was frequently high and especially so in Cases 222, 205, 204, and 219, where the blood samples were taken shortly before death.

Cholesterol.—Practically normal throughout.

Fat.—In the plasma this value was, with two exceptions, much above the normal. In the corpuscles it was frequently abnormally high and the high values were most marked in those cases with the most severe symptoms.

Total Fatty Acids: Lecithin.—In the plasma this ratio was generally much above normal (due to excess of fat). In the corpuscles it was occasionally high.

Lecithin: Cholesterol.—This ratio was normal in most cases in both plasma and corpuscles but high in those cases with more severe symptoms.

Total Lipoids.—Generally above normal.

The percentage of corpuscles was generally below normal and in some of the severe cases very much below, as in the last two of the series.

The plasma in this series of cases was free from visible fat although occasionally muddy with material in coarse suspension.

The most marked abnormalities observed in the blood lipoids in this series were then as follows:

1. High values for total fatty acids in both plasma and corpuscles.
2. High fat in the plasma (with occasional high values in the corpuscles) which in general was most marked in those cases with more severe symptoms.
3. Frequent high values for lecithin in the corpuscles, which were very marked in fatal cases where the samples were taken shortly before death.
4. High values for total lipoids in the plasma.

The high total fatty acids in the plasma and corpuscles and the high lecithin in the corpuscles with normal cholesterol are the conditions observed in the blood in alimentary lipemia (13) and

therefore strongly suggest that the abnormalities observed in the lipoids in nephritis are due to a retarded fat assimilation in the blood. The extent of the abnormality would in that case depend on at least three factors—the extent of the retardation, the amount of fat present in the blood when the process began, and the amount of fat entering the blood from the alimentary canal. The great variations in the blood lipoids in nephritis reported in the literature are explainable as the result of differences in these factors.

On the basis of work already done on the blood lipoids it seems possible to distinguish between "acute" and "chronic" disturbances in the blood lipoids—acute disturbances such as occur in alimentary lipemia, which are characterized by increased fat and lecithin, and chronic disturbances characterized also by increased cholesterol, of which the best example is diabetes. The excess of lipoids in both these examples frequently results in milkiness of the plasma. The abnormalities in nephritis observed in this work would, on this basis, be classified as acute disturbances, but there is some evidence in the literature to show that the condition may become chronic with high cholesterol values. The best example of this chronic condition reported is that of Müller already noted (9). In this case the distribution of the blood lipoids was similar to that found in diabetes—high fat (glycerides) with cholesterol increased almost parallel with the fat, while lecithin, although much above the normal value, had still not increased to anything like the extent of the other two. The plasma in this case was milky. Other cases reported in the literature had high cholesterol and lipemia (6, 7). No milkiness of the plasma was observed in any of the series reported in this paper although the plasma was occasionally muddy from matter in coarse suspension, which was probably not fat.

As to the cause which produces these disturbances in the lipoids in nephritis, the most frequent other condition in which abnormalities of the blood lipoids are common is diabetes and a prominent symptom of severe diabetes is "acidosis"—a decreased "alkali reserve" in the blood. Recent investigations (14) have established the fact that acidosis is frequently a feature of severe nephritis, and in most of the cases of this series acidosis was probably present, as evidenced by the dyspnea, low carbon dioxide

tension in the alveolar air, and coma. Since an adequate alkalinity of the blood and tissues is necessary for their normal functioning it seems very probable that the retardation of fat assimilation found in nephritis is one manifestation of a general phenomenon brought about by a decreased blood and tissue alkalinity.

SUMMARY.

The abnormalities in the blood lipoids in severe nephritis were found to be high fat in plasma and corpuscles and high lecithin in the corpuscles. The cholesterol values were practically normal. These abnormalities are the same as are found in alimentary lipemia and for this reason are regarded as the result of a retarded assimilation of fat in the blood, which in turn is thought to be one manifestation of a general metabolic disturbance brought about by a lowered "alkali reserve" of the blood and tissues.

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THE DYNAMICS OF THE PROCESS OF DEATH.

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(Received for publication, July 25, 1917.)

The writer has found that by measuring the electrical conductivity of tissues placed in toxic solutions the process of death can be followed in the same manner as the progress of a reaction *in vitro*.

Studies on a considerable variety of toxic solutions have shown that in them death proceeds as a monomolecular reaction. In the case of *Laminaria* in NaCl, it was observed[1] that the reaction behaves as if it were "inhibited" at the start, as shown by the fact that the velocity constant was fairly regular except at the start, where it was clearly below the average value. At that time the writer was unable to suggest a satisfactory explanation of this "inhibition." Later studies[2] have afforded a clue to the explanation. In these studies it was found necessary to assume that death should be regarded as a series of reactions of the type $O \rightarrow A \rightarrow M \rightarrow B$.

It is assumed that M is a substance which determines the normal permeability and electrical resistance (and perhaps other normal properties) of the protoplasm. As long as the tissue remains in its normal environment M is formed as rapidly as it is decomposed but when the tissue is placed in NaCl (which is toxic for *Laminaria*) the reaction $O \rightarrow A$ ceases, while the reactions $A \rightarrow M \rightarrow B$ proceed at an increased rate. As a result the quantity of M decreases to zero, at which point the tissue is regarded as dead. This point can be determined by measuring the electrical conductivity of the tissue as can any intermediate point (*e. g.*, half dead, etc.).

[1] The methods of measurement and of calculation are explained in *Science*, 1914, xxxix, 544.

[2] Osterhout, W. J. V., *Proc. Am. Phil. Soc.*, 1916, lv, 533.

We may assume for convenience that in sea water (the normal environment of *Laminaria*) the concentrations[3] of A and M are constant at 8.853 and 0.2951 respectively, and that on transferring to NaCl the velocity of the reactions $A \rightarrow M$, and $M \rightarrow B$, increases to 0.018 and 0.540 respectively. We assume that these reactions are monomolecular and irreversible (or practically so). We can then calculate the amount of M (this amount will for convenience be called y) at any time, T, after the tissue is transferred to the solution of NaCl.

As explained in a previous paper,[2] we may employ for this purpose the formula:

$$y = 0.2951\ (e^{-K_2T}) + 8.853\left(\frac{K_1}{K_2 - K_1}\right)(e^{-K_1T} - e^{-K_2T})$$

in which y is the amount of M, T is the time of exposure to the solution of NaCl, e is the basis of natural logarithms, and K_1 and K_2 are the velocity constants of $A \rightarrow M$ and $M \rightarrow B$ respectively.

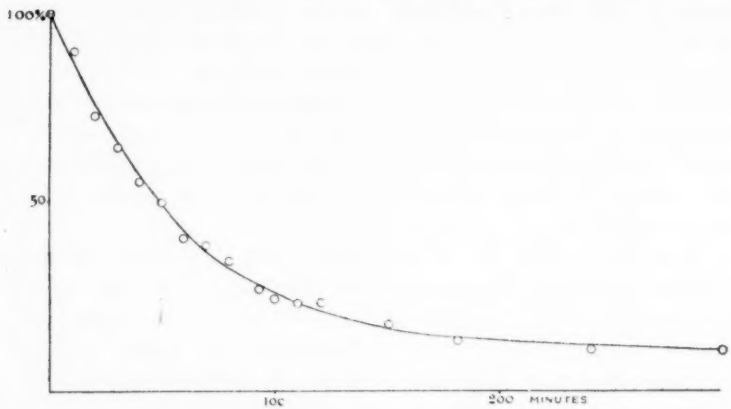


FIG. 1. Electrical resistance of *Laminaria* in a solution of NaCl. Experimental values (o, o) and calculated curve.

The results of a series of such calculations are given in Table I, together with the values obtained in a recent series of experiments. The calculated and observed values are plotted in Fig. 1. It will be seen that the agreement is very satisfactory.

[3] These values were used because they had been employed in previous calculations. The values of y are multiplied by 305 and 10 is added.

TABLE I.*

Net Electrical Resistance of Laminaria in NaCl 52 M. The Resistance in Sea Water (the Normal Environment) Is Taken as 100 per Cent.

Time.	Resistance.		K_3	
	Observed.	Calculated.	From observed values.	From calculated values.
min.	*per cent*	*per cent*		
10	87.50	87.76	0.0065	0.0064
20	73.01	74.96	0.0077	0.0071
30	62.51	64.26	0.0078	0.0073
40	55.30	55.32	0.0075	0.0075
50	48.81	47.86	0.0073	0.0075
60	40.21	41.62	0.0079	0.0076
70	36.79	36.41	0.0075	0.0076
80	32.41	32.06	0.0076	0.0076
90	27.52	28.43	0.0079	0.0077
100	24.69	25.39	0.0079	0.0077
110	23.00	22.86	0.0076	0.0077
120	22.82	20.74	0.0071	0.0077
150	16.51	16.26	0.0076	0.0077
180	14.54	13.65	0.0072	0.0077
Average.....			0.0075	0.0075

* All readings were made at 15°C., or corrected to this temperature.

If we were unaware that this curve represented two consecutive reactions, and supposed it to represent a simple monomolecular reaction ($M \rightarrow B$), we should calculate its velocity constant by the usual formula:[4]

$$K_3 = \frac{1}{T} \log \left(\frac{a}{a - x} \right)$$

If we make this calculation, employing for this purpose the calculated values given in the third column of Table I, we obtain the values of the velocity constant K_3 given in the fifth column of Table I.

It is evident from an inspection of these values that the velocity constant K_3 falls below the average value at the start.

The amount by which it falls below the average value will depend on the relation $K_1 \div K_2$. When K_1 and K_2 are nearly

[4] Common logarithms are used for convenience. We put $a = 100 - 10$ and $a - x = y - 10$.

equal, the velocity constant falls a good deal below the average value at the start, but as the difference between them is increased the velocity constant K_3 will be found to fall less and less below the average value at the start.[5] This is easily shown by assuming various values[6] of K_1 and K_2.

From this it follows that we can tell something about $K_1 \div K_2$ from the experimental values of K_3. It is evident that in the present case the experimental values of K point to the relation $K_2 \div K_1 = 30$ (or $K_1 \div K_2 = 30$). This relation was actually assumed by the writer in a previous paper in order to fit, not the NaCl curve, but antagonism curves[7] in various mixtures of $NaCl + CaCl_2$. It is therefore a striking confirmation of the general correctness of the underlying assumption that we are also able by this assumption to fit the NaCl curve so closely.

In general, where a chemical reaction is slower at the start than is expected, we may suspect that we have to do, not with a simple reaction, but with consecutive reactions of the kind here described.[8]

This explanation also applies to a considerable number of other cases of toxic action.

It is of interest that in all these cases death behaves as a reaction which is continually going on but at a very slow rate until accelerated by the toxic agent. We have assumed this acceleration to consist partly in the increase of the velocity constant and partly in the stopping of the reaction $O \rightarrow A$, causing a decrease in the substance (M) to which normal permeability (and perhaps other normal properties) are due.

It may prove to be generally true that death behaves as a monomolecular reaction, more or less inhibited (or accelerated)

[5] It should be noted that we get the same result (as regards K_3 falling below the average at the start) when $K_1 \div K_2 = 30$ as when $K_2 \div K_1 = 30$. With certain relations of $K_1 \div K_2$ the constant K_3 may be above the average value at the start.

[6] When the values of K_1 and K_2 are changed, the concentrations of A and M must also be changed in such a way that Conc. $A \div$ Conc. $M = K_1 \div K_2$ if we wish the concentrations of A and M to remain constant in the normal environment.

[7] The manner in which the relation $K_1 \div K_2$ influences the forms of these curves cannot be discussed in this paper.

[8] Mellor, J. W., Chemical Statics and Dynamics, London, 1909, chapt. vi.

at the start. The assumption of consecutive reactions affords an explanation not only of the inhibition (or acceleration) at the start but also of the fact that up to a certain point the reaction appears to be reversible. The latter fact will be fully discussed in a subsequent paper.

SUMMARY.

In the case here described, death proceeds as a monomolecular reaction which is somewhat "inhibited" at the start. This is easily explained if consecutive reactions are involved in the process of death. This explanation also applies to many other cases of toxic action.

In all these cases death behaves as a reaction which is continually going on and which is accelerated by the toxic agent.

THE STRUCTURE OF YEAST NUCLEIC ACID.

By P. A. LEVENE.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, July 26, 1917.)

Levene and Jacobs have formulated the structure of yeast nucleic acid as a tetranucleotide. The facts that led up to their formulation were:

1. The formation of four nucleosides on neutral or ammonia hydrolysis.

2. The formation of simpler nucleotides on hydrolysis with dilute mineral acids.

3. The presence of a phosphorus to nitrogen ratio which agreed quite well for the tetranucleotide theory.

4. The ratio of amino to total nitrogen in nucleic acid was in harmony with the ratio required by the four bases, guanine, adenine, uracil, and cytosine.

The mode of linkage between the individual nucleotides was at that time not determined, and in the graphic formula representing the nucleic acid the linkage of the nucleotides was of a provisional character and arbitrary in nature.

Following that, the studies of Levene and Jacobs on thymus nucleic acid led to methods which permitted the separation and the study of individual mono- and dinucleotides composing the complex nucleic acids. Levene and Jacobs then returned to the study of yeast nucleic acid, applying the experience gained on the thymus nucleic acids. They then directed their attention to the nucleotides obtained by acid hydrolysis of the yeast nucleic acid. A large quantity of the material was prepared and transformed into the brucine salt. Other work, however, made a demand on their energies, and the work on the nucleotides was somewhat neglected. However, in course of the present academic year the work has been resumed. There were on hand 125.0 gm. of the brucine salts when the work was begun.

Meanwhile Dr. Walter Jones and his coworkers published several important publications on the structure of yeast nucleic acid. The basis of their work is the conception of the nucleic acid molecule as expressed by Levene and Jacobs.

The work of Jones deals specifically with the mode of linkage between individual nucleotides; Jones accepts a tetraribose of the structure $((C_5H_{10}O_5)_4 - 3H_2O)$ as the nucleus of the molecule. In this nucleus all the carbonyl groups of course must be free, since this is demanded by the existence of nucleosides. Dr. Jones bases his conclusion on two arguments: one is a proof by analogy; namely, the wide distribution of polysaccharides in nature; the other is the discovery by him of dinucleotides in which four of the hydroxyls of the phosphoric acid are free. The first argument does not seem valid, since according to present knowledge all the polysaccharides in nature have a glucosidic structure, and the one assumed by Jones to be present in the molecule of nucleic acid can be constructed only through ether linkage. Just because a proof by analogy is lacking, all the greater rigor is required from the experimental evidence.

There have appeared three papers by Jones and his coworkers dealing with the subject. In one, Jones and Richards claim to have cleaved the molecule of yeast nucleic acid into two large fractions, guanine-cytosine dinuclectide, and adenine-uracil dinucleotide. However, the authors admit that the dinucleotides were not isolated in pure form.

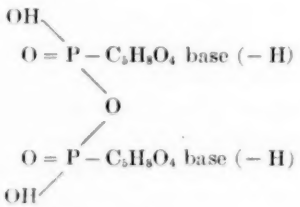
In a second paper Jones and Germann claim to have accomplished by ammonia hydrolysis the same cleavage as Jones and Richards had accomplished by enzymes. However, from the fraction named guanosine-cytosine only guanylic acid was obtained. The adenine-cytosine fraction analyzed for the dinucleotide and a brucine salt obtained from it analyzed satisfactorily for the same substance. The uracil nucleotide was not traced.

In a third paper Jones and Read, following closely the directions of Levene and Jacobs, prepared the pyrimidine nucleotide fraction previously obtained by these authors. Jones and Read converted it into the brucine salt and found the analytical data of this substance to agree with that of cytosine-uracil dinucleotide.

Thus the principal argument of Jones and his coworkers in favor of the dinucleotide structure of their substances is based on the analysis of the brucine salts. The alkaloid brucine was introduced into the study of nucleotides by Levene and Jacobs. The advantage of this reagent, as seen by them, consisted in the fact that it permitted fractionation of the brucine salts of nucleotides not only out of water, but also out of ethyl or methyl alcohol. The separation of the cytosine and thymine nucleotides of the thymus nucleic acid was based on this property of the brucine salts. Not much importance was attributed to the analytical data of the brucine salts. Indeed, Jones did not exaggerate the value of the analytical data of the brucine salts when he criticized the work of Tannhauser. Employing the same ammonia hydrolysis Tannhauser thought he had isolated a trinucleotide. Jones took exception to the conclusion on the basis of the fact that the percentage composition of the brucine salt was not much different from that of the brucine salt of guanylic acid.

Levene and Jacobs, in their previous work on the brucine salts of nucleotides, formulated the following requirement as a test of the individuality of a nucleotide. First, a constant composition of the brucine salt on fractional crystallization, and second, a conversion of the brucine salt into a barium salt, which furnished analytical data agreeing with the theory for the assumed substance. This seemed the minimum requirement.

But even admitting that all the dinucleotides of Jones and coworkers actually exist, does this fact necessarily force the conclusion of a tetraribose nucleus? There are theoretically possible not only two but six ways of linkage between two nucleotides. They are:



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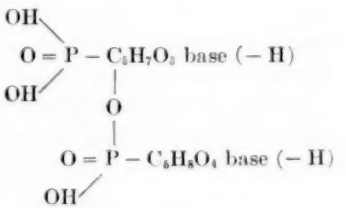

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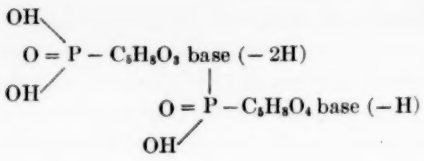

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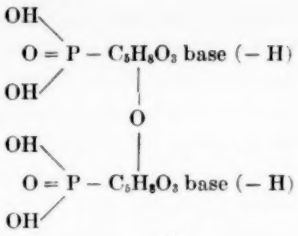

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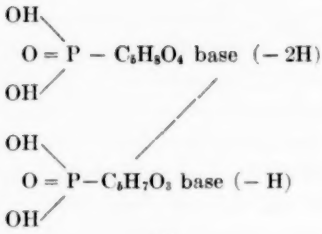

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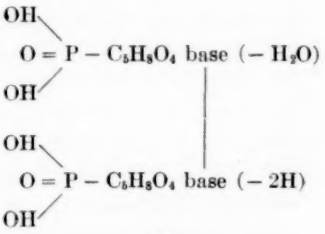


VI

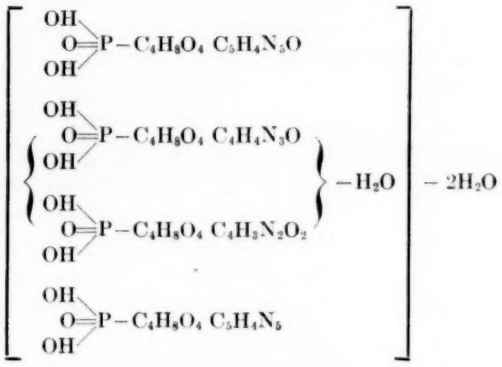
Forms III and V are possible of existence only when at least one of the two bases has two NH groups in the molecule and form VI when one base has two NH groups and the other, one NH and one OH groups.

In the first three forms of dinucleotides there are less than four readily ionizable hydrogen atoms. They should form di- or tribasic salts. But we have seen in guanylic acid the readiness with which a nucleotide forms a basic salt. Furthermore, dinucleotides of type II were shown to be present in thymus nucleic acid.

The remaining three may all function as tetrabasic acids. Forms IV and V may be regarded as the two most probable structures for dinucleotides forming tetrabrucine salts. A decision between these two alternatives must be based on either experimental data or on valid theoretical reasoning.

These possibilities must be particularly borne in mind, because of the two following facts: first, in purine nucleotides the phosphoric acid is less firmly linked to the nucleoside than in pyrimidine nucleotides; and second, pyrimidine nucleotides form a dinucleotide more resistant toward hydrolytic action of acids than the purine nucleotides. The latter fact was first shown by

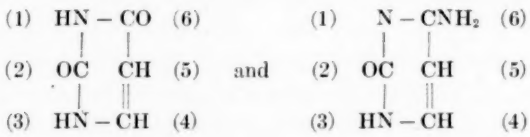
Levene and Jacobs for thymus nucleic acid, and is now shown to hold also for yeast nucleic acid. These differences in behavior are surely due to differences in structure. If a diribose is proven to exist in all dinucleotides, then the difference will have to be explained by the differences in the position of the hydroxyl groups which form the oxidic linking of two molecules of ribose. The final decision on the details of the structure of dinucleotides has to be reserved. All these considerations make it a difficult task to express the structure of yeast nucleic acid in a graphic formula without any arbitrary elements. If such a one is desired, it can only be expressed for the present in the following manner.



In the present communication it is desired to report on the cytosine-uracil dinucleotide prepared some time ago. The crude material was prepared nearly 5 years ago and the dinucleotide was isolated about 4 months ago. The result was not communicated because more light on the mode of linkage was desired. However, because of the recent publication of Jones and Read, our results are now presented. By means of fractional extraction with methyl alcohol the crude brucine salt was separated in several fractions, each different in composition. The fractions that came closest in their composition to that of the brucine salt of the cytosine-uracil dinucleotide were converted into a crystalline barium salt. The analytical data of the substance agreed with the theory for the assumed dinucleotide. The ratio of the amino to total nitrogen of the salt agreed with the theory for the barium salt of the dinucleotide. The melting

point of our brucine salt was 200°C. (corrected). The physical constants of our substance are slightly different from those of Jones and Read. This is not surprising since Levene and Jacobs have already shown that the crude barium salt is altered in composition by reprecipitation, and it is now also shown that various fractions of the crude brucine salt vary in composition. Furthermore, we are not aware of a single instance in which a partial hydrolysis leads to products of only one phase of hydrolysis. As a rule, under such conditions, substances of different degrees of cleavage are formed. Indeed, in the partial hydrolysis of thymus nucleic acid Levene and Jacobs have shown the presence simultaneously of thymine-cytosine dinucleotides and of the mononucleotides of the same bases. Whether the substance of Jones and Read or ours is nearest to the pure dinucleotide remains to be established.

As regards the mode of linkage of this dinucleotide, structure IV may perhaps be accepted, however, not with absolute certainty, as structure V cannot be excluded without further proof. However, if structure V were to be accepted it would necessitate the acceptance of a union between a hydroxyl group of the sugar and one of the nitrogen atoms of the base. Taking into consideration the structure of the two bases uracil and cytosine,



and further, taking into consideration the fact that in cytosine the NH_2 group is unsubstituted and that therefore cytosine cannot serve as a connecting link between two nucleotides, then uracil would have to be accepted as the bridge between two riboses of the dinucleotide. Future work will have to decide between the two structures. This applies also to the pyrimidine dinucleotide from thymus nucleic acid.

EXPERIMENTAL.

The bulk of the material was the brucine salts prepared about 5 years ago from the barium salts described by Levene and Jacobs.

50.0 gm. of the brucine salt were extracted with 1,500 cc. of boiling methyl alcohol. The insoluble part was successively extracted until the insoluble residue did not perceptibly lose in weight on two successive extractions. The alcoholic extracts on standing at 25°C. formed a crystalline deposit. This was filtered, and the filtrate concentrated to dryness. The residue was recrystallized out of water. Out of 50.0 gm. there remained 14 gm. as the difficultly soluble part. This fractionation was carried out on two lots of 50.0 gm. each of the old material, and on two smaller samples prepared in course of this year. In the following table is given a summary of the analytical data of the various samples.

Sample No.	C	H	N	P	M. P.	$[\alpha]_D^{25}$
Insoluble.						
1	56.56	5.89	6.55	2.85	Contracted 178°C. Decomposed 215°C.	$\frac{-0.02\times10.0}{1\times0.05}=-4.0°$
2	56.20	6.09	6.62	2.64		
3	56.72	6.18	6.11	3.17		
Soluble.						
4	58.18	6.37	8.05	2.70	Contracted 185°C. Decomposed 200°C.	$\frac{-0.05\times10.0}{1\times0.05}=-10.0°$
5	58.99	6.68	7.89	2.79	Contracted 185°C. Decomposed 200°C.	$\frac{-0.13\times5.0}{1\times0.05}=-13.0°$
6	58.47	6.93	9.05	2.71		
Theory for dinucleotide	59.36	6.73	8.19	2.80		

Samples 5 and 6 were dissolved in warm water with the aid of a slight excess of ammonia and shaken in a separatory funnel with chloroform to remove all brucine. To the brucine-free solution barium hydroxide was added in slight excess over the quantity theoretically required to neutralize the acidity of the nucleotide, and the solution was repeatedly evaporated to dryness under diminished pressure until all ammonia was removed. The residue

was then dissolved in water and neutralized to litmus with sulfuric acid, filtered from barium sulfate, and concentrated under diminished pressure to a very small volume until an insoluble white precipitate began to form. On cooling, the precipitate increased. It was filtered, dissolved to a clear solution, and again concentrated to a small volume. This time a granular precipitate settled out which under the microscope consisted of striated plates and needles.

0.0955 gm. substance required for neutralization 4.93 cc. 0.1 N acid.
0.1909 " " gave 0.0426 gm. $Mg_2P_2O_7$.
0.0955 " " " 0.0464 " $BaSO_4$.
0.0966 " " " 0.0798 gm. CO_2 and 0.276 gm. H_2O.
0.0020 " " " in Van Slyke micro-apparatus 0.59 cc. N at 14°C. and 748 mm.

	Calculated for $C_{18}H_{23}N_5O_{16}P_2Ba_2+2H_2O$:	Found:
C	22.69	22.53
H	2.62	3.19
N	7.63	7.23
P	6.76	6.24
Ba	28.72	28.59
NH_2N	1.53	1.70

The optical rotation of the substance in 2.5 per cent HCl was:

$$[\alpha]_D^{25} = \frac{+0.50° \times 2.5}{1 \times 0.100} = +12.5° \text{ for the barium salt} = +18.52° \text{ for the free dinucleotide.}$$

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THE REMOVAL OF NITRIC ACID FROM SOLUTIONS OF ORGANIC COMPOUNDS.

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(Received for publication, July 26, 1917.)

The lack of a convenient method for removing nitric acid has restricted to a minimum its use in organic and biological chemistry. In this laboratory we have felt the disturbing presence of nitric acid particularly in the preparation of the salts of anhydrotalonic and anhydrogalactonic acids, and of similar substances.

In inorganic analysis several methods for removing nitric acid are used. The majority of them are based on the process of reduction. Unfortunately the reduction is frequently carried out under conditions that would act destructively on the organic material, if any were present. The problem as it presented itself to us consisted in the selection of a method of reduction which could be carried out in a solution with a reaction in the neighborhood of neutrality and at ordinary temperature. Besides it was desired to select such reagents as were readily removable from the solution.

Of the reducing agents generally recommended for this purpose the following were employed: zinc dust, zinc-copper couple,[1] iron and sulfuric acid,[2] and aluminum amalgam.[3] By means of zinc dust or zinc-copper couple in solutions approaching neutrality the reduction proceeded very slowly and never reached completion. On the other hand, by means of aluminum amalgam the reduction is completed in 6 hours. When the process of reduction of nitric acid by means of aluminum amalgam was recommended as a method for the quantitative determination of nitric acid, it was assumed that the acid was reduced completely

[1] Williams, M. W., *J. Chem. Soc.*, 1881, xxxix, 100, 144.

[2] Alberti and Hempel, *Z. angew. Chem.*, 1892, 101.

[3] Ormandy, R., and Cohen, J. B., *J. Chem. Soc.*, 1890, lvii, 811.

to ammonia. Under the conditions here described only about 40 per cent of the nitric acid was reduced to ammonia; the remaining 60 per cent escaped the solution undoubtedly in the form of lower oxides of nitrogen. In this connection it was interesting to note that the rate of disappearance of nitric acid was much higher than that of ammonia formation, thus showing that in the course of the reduction the proportion of the lower oxides compared with that of N_2O_5 was continually increasing. Because of this it was deemed important to show experimentally that when the solution became free from nitric acid it was also free from N_2O_3.

Theoretically one could not expect to find an absence of nitric acid where nitrous acid was present, since, in aqueous solution, nitrites always assume the following equilibrium:

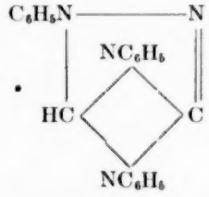
$$3MNO_2 + H_2O = MNO_3 + 2NO + 2MOH.$$

However, to remove all possible doubt, the reduced solution was analyzed for both nitric and nitrous acids.

Nitric acid was estimated by means of "nitron."[4] At the end of the experiment a test for nitric acid was also made by means of brucine. The test was always negative.

The absence of nitrous acid was shown by means of a solution of potassium permanganate. After standing with the reduced solution for 1 hour no reduction of the permanganate solution could be detected. Hence it was proven that aluminum amalgam could be employed conveniently for removing nitric and nitrous acids.

[4] *Diphenyl-endanilo-dehydrotriazol,* with which it forms an insoluble precipitate.



Busch, M., *Ber. chem. Ges.*, 1905, xxxviii, 681. Gutbier, A., *Z. angew. Chem.*, 1905, xviii, 494.

When the process is applied to the removal of nitric acid from organic mixtures it becomes necessary also to remove the introduced reagents or their transformation product. The following procedure was adopted.

1. The acidity of the solution is determined by titration of a small sample and the solution is then neutralized by means of barium hydroxide.

2. About 2 gm. of freshly prepared aluminum amalgam are added for each gm. of nitric acid. The reduction is allowed to proceed for 8 hours or over night. The solution is aerated during the entire time of reduction.

3. The mixture is filtered from the mercury and aluminum. A slight excess of barium hydroxide is then added to the filtrate, and the mixture is concentrated under diminished pressure, to remove ammonia. Generally the process is completed after the evaporation has been repeated twice.

4. The barium is removed quantitatively and the filtrate is ready for further operations.

EXPERIMENTAL.

Determination of Nitric Acid with Nitron.—The solution of barium nitrate containing the equivalent of about 100 mg. of nitric acid was acidified with sulfuric acid and filtered from barium sulfate. To the filtrate 12 to 15 cc. of 10 per cent nitron solution in 5 per cent acetic acid were added and then the mixture was cooled in an ice bath. The precipitate was transferred to a weighed alundum crucible, washed with ice cold water, dried at 105–110°, and weighed. The weight of nitron nitrate $\times$ 0.168 = nitric acid.

Determination of Ammonia.—Ammonia was determined by distilling aliquot portions of the solution, made alkaline, into 0.1 N acid. In those experiments in which the nitrate solutions were aerated during the reduction, the diluted acid solution into which the air was passed was added to the sample taken from the reaction flask, so that the determination in all experiments represents the total quantity of ammonia which was formed during the period indicated.

Aluminum Amalgam.—Sheets of aluminum foil about 4 by 6 inches are passed through a flame to remove the grease and im-

mersed in a shallow bath of about 3 per cent solution of mercuric chloride. In a few minutes the surface of the foil is covered with mercury. The foil is immediately washed in running water and is at once transferred to the nitrate solution.

Reduction of Nitrate Solutions.—Dilute solutions of barium nitrate were placed in flasks with aluminum amalgam. These flasks were fitted with rubber stopper and tubing so that the ammonia which might be generated would be collected in wash bottles containing dilute acid. In some experiments the ammonia was drawn into the dilute acid by a slow current of air.

Experiment 1.—4 gm. of barium nitrate were dissolved in 200 cc. of water and 2 gm. of aluminum amalgam were added and allowed to remain for about 2 hours without aeration. After 12, 36, and 60 hours, samples were withdrawn for analysis. The results are shown in Table I.

TABLE I.

Time.	Theory NH_3 obtainable.	NH_3 found.	Theory.
hrs.	*gm.*	*gm.*	*per cent*
0	0.520		
12		0.0774	14.9
36		0.1449	27.9
60		0.0483	37.6

Experiment 2.—8 gm. of barium nitrate were dissolved in 400 cc. of water and 4 gm. of aluminum amalgam were added. This solution was aerated and the ammonia was determined after 12, 36, and 60 hours. The results are given in Table II.

TABLE II.

Time.	Theory NH_3 obtainable.	NH_3 found.	Theory.
hrs.	*gm.*	*gm.*	*per cent*
0	1.0400		
12		0.2734	27.0
36		0.1049	31.63
60		0.0219	38.00

Experiment 3.—2 gm. of barium nitrate were dissolved in 100 cc. of water, 1 gm. of aluminum amalgam was added, and aerated

for 14 hours, after which time the nitric acid test with nitron was negative as well as with brucine.

Experiment 4.—8 gm. of barium nitrate were dissolved in 400 cc. of water and 6 gm. of aluminum amalgam were added. After 4 hours the nitric acid test with the nitron and brucine was negative.

Experiment 5.—50 cc. of 2.3 per cent nitric acid and 1 gm. of aluminum amalgam were allowed to stand for 16 hours. After 4 hours, 15.9 per cent acid was not reduced and after 16 hours 4.6 per cent nitric acid remained.

Experiment 6.—10 gm. of barium nitrate were dissolved in 500 cc. of water and 7 gm. of aluminum amalgam were added. Then additional amalgam was added every 2 hours as indicated in the table, and at each 2 hour interval a sample of the liquid was withdrawn for the determination of nitric acid and ammonia. As Table III shows, no nitric acid remained after 6 hours although only 40 per cent of the nitric acid was obtained in the form of ammonia.

TABLE III.

	Nitric acid determined with nitron.					Ammonia (NH_3).				
Time.	Aluminum amalgam.	Present.	Reduced.	Present.	Reduced.	Calculated from HNO_3 present.	NH_3 determined.	Total produced.	NH_3 obtained during each interval.	NH_3 obtained from beginning.
hrs.	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*
0	7.0	5.660	0.000	100.0	000.0	1.527				
2	3.0	1.392	4.268	24.0	76.0		0.1205	0.1205	7.90	7.60
3	2.0	0.558	5.102	9.8	90.2		0.1475	0.2680	9.68	17.60
4	0.5	0.0045	5.655	0.79	99.21		0.1665	0.4345	10.90	28.50
5	0.5	Trace.	Nearly all.	Trace.	99.99		0.1904	0.6249	12.48	40.96
6	0.5	None.	5.660	None.	100.00					

Experiment 7.—10 gm. of barium nitrate were dissolved in 500 cc. of water and 7 gm. of aluminum amalgam were added. The solution was aerated and allowed to stand for 14 hours. The mixture was filtered and the filtrate tested for nitric and nitrous acids. Nitric acid was found absent both with brucine and nitron. 20 cc. of 0.1 N potassium permanganate acidified with

sulfuric acid were added to 20 cc. of the filtrate and the unused permanganate was titrated, according to the method of Volhard, with potassium iodide. No nitrous acid was found.

THE PREPARATION OF LYXOSE.

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Of the methods for preparation of lyxose the one introduced by Ruff and Ollendorff[1] is the most convenient. This consists in the oxidation of calcium *d*-galactonate by means of hydrogen peroxide, using ferric acetate as a catalyst. The details of the directions as given by Ruff and Ollendorff are not sufficient for the preparation of this pentose on a large scale. In the course of the past year over 3,000 gm. of the sugar were prepared in this laboratory, and the process has been gradually improved so that finally a yield of 195 gm. of pure crystalline lyxose was obtained from 1,500 gm. of calcium galactonate. The conditions as finally adopted are reported here in the hope that they may prove useful to other workers.

EXPERIMENTAL.

500 gm. of calcium galactonate were dissolved in 2 liters of boiling water, and 3 liters of 3 per cent hydrogen peroxide added. The solution was cooled to about 35° and 75 cc. of ferric acetate solution[2] were added, which soon caused a vigorous reaction. After the reaction was completed, which was indicated by the solution acquiring a deep purple color, it was allowed to cool. The solution was filtered and evaporated in vacuum to about 1,200 cc. To the concentrated solution 4 liters of 95 per cent alcohol were added with constant stirring. This precipitates a gummy mass which is hard to handle but if a current of air is blown through the suspension for a short time, all the gummy particles settle out, leaving a clear solution. This solution was

[1] Ruff, O., and Ollendorff, G., *Ber. chem. Ges.*, 1900, xxxiii, 1798.

[2] National Formulary, 3rd edition, Baltimore, 1906, p. 219.

filtered with suction. The gum remaining in the jar, which was drained as dry as possible, and the precipitate on the filter, were then dissolved in about 900 cc. of hot water. This may be readily accomplished in 4 or 5 minutes by heating the mixture, with constant stirring, to about 60–65° by means of live steam. After the gum had dissolved, the liquid was cooled to room temperature and precipitated with 4 liters of 95 per cent alcohol, as above.

Three lots of 500 gm. each were treated in this way and the combined residues from them were reoxidized in the following manner: They were dissolved in several liters of hot water, allowed to cool, and filtered. The filtrate was evaporated to dryness *in vacuo* to remove all the alcohol. The residue was then dissolved in about 2 liters of hot water by means of live steam and 5 liters of hydrogen peroxide were added. The solution was cooled to 35° and 80 cc. of ferric acetate solution were added. After the reaction was complete the solution was filtered and concentrated, then precipitated with 95 per cent alcohol as previously described. The residues were dissolved in 1 liter of water and again precipitated with 4 liters of 95 per cent alcohol.

The combined alcoholic extracts resulting from the above procedure were then evaporated *in vacuo* to 1 liter. 95 per cent alcohol was added with constant stirring until a permanent precipitate was formed; about 1.5 liters of alcohol were required. This solution was poured into 9 liters of absolute alcohol with constant agitation. The precipitate formed was filtered off, drained as dry as possible, and the filtrate evaporated *in vacuo* to a thick syrup (about 700 cc.). This syrup was taken up in 8 liters of absolute alcohol and 3.5 liters of dry ether *slowly* added with constant stirring, which precipitates a further quantity of calcium salts and other reaction products. The filtered solution was evaporated *in vacuo* to 500 cc., seeded with a few crystals of lyxose, and allowed to crystallize in a desiccator. Often the syrup can be made to crystallize spontaneously without seeding by scratching the inside of the beaker.

The crystals were filtered with suction and washed first with absolute alcohol and then with dry ether. The yield was generally 150 to 165 gm. of pure dry sugar. Lyxose may be readily

recrystallized with little loss from four to five parts of boiling absolute alcohol. No attempt was made to work over the mother liquors from the crystallization of lyxose, as it was found that they could be used directly for the preparation of lyxosimine. The syrup gave a yield of lyxosimine corresponding to about 55 or 60 gm. of lyxose. Yields of pure crystalline lyxose from lots of 1,500 gm. of calcium galactonate have been obtained as high as 195 gm., but in these cases a corresponding diminution of lyxosimine has been obtained from the mother liquors, so that almost invariably the total yield corresponded to about 210 gm. of lyxose from 1,500 gm. of calcium galactonate.

CHONDROSAMINE AND ITS SYNTHESIS.

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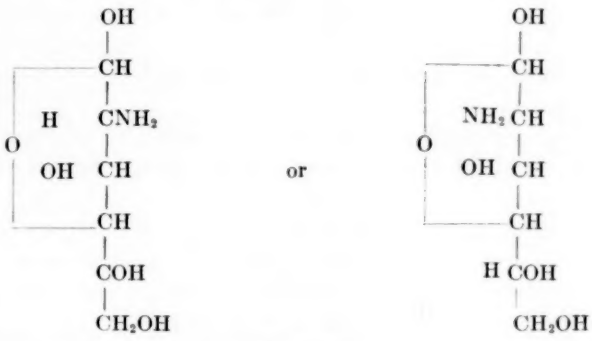
In previous publications[1] the conclusion was reached that chondrosamine had the structure of one of the two lyxohexosamines:



The relationship of chondrosamine to galactose was originally based on the following facts:

Chondrosamine formed with phenylhydrazine an osazone indistinguishable from galactosazone. Chondrosamine, on oxidation with bromine or with mercuric oxide, formed chondrosaminic acid. This acid on oxidation with nitric acid (subsequent to deamination) yielded anhydromucic acid. On the other hand direct oxidation of chondrosamine (subsequent to deamination) led to an optically active dicarboxylic acid which was assumed to be anhydrotalomucic acid. Furthermore, chondrosamine on oxidation with bromine or with mercuric oxide (subsequent to deamination) gave rise to anhydrotalonic acid. Chondrosaminic acid under the same treatment formed the isomeric anhydro-

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1913, xv, 159; 1914, xviii, 127, 240; 1915, xx, 434.

galactonic acid. In a measure the proof seemed sufficient to establish the configuration of the new aminohexose. Yet a scrutiny of the evidence makes it clear that only one point of evidence was absolutely beyond dispute; that is, the identity of the chondrosamine osazone with galactosazone. True, the two anhydrotetrahydroxyadipic acids, one obtained from chondrosamine and the other from lyxohexosaminic acids, seemed identical. However, this conclusion was based on their melting points being identical and on the absence of optical activity in one and the other. These points may be considered ample proof, but additional evidence was desirable. Again the anhydrotalonic and anhydrogalactonic acids had been prepared only in form of their brucine salts, and although the differences in their optical rotation were in full agreement with the assumed structure of the two salts, yet more direct evidence in support of the assumption was much wanted.

The most convincing proof possible would be of course the synthesis of all the substances derived from chondrosamine, using lyxose as the starting material. The synthesis of the sugar has now been accomplished, and with it for every known derivative of the natural sugar a corresponding derivative of the synthetic substance has been obtained. Thus the problem of the configuration of chondrosamine is definitely solved.

Chondrosamine Hydrochloride.—This was prepared last year by the reduction of lyxohexosaminic acid. Its optical rotation was then found to be $[\alpha]_D^{\circ} = +62.69$ to $+91.10°$. The rotation of chondrosamine hydrochloride was determined by Levene and La Forge, $[\alpha]_D^{\circ} = +129.50$ to $93.82°$. This difference in rotation and the fact that chondrosaminic acid differed in its rotation from the synthetic lyxohexosaminic acid led originally to the conclusion that the natural and the synthetic sugars were epimers. However, the fact that the rotation of the two sugars reached the same value at the state of equilibrium suggested the possibility that they were α and β forms of the same sugar. Hence the rotation of the recently prepared chondrosamine hydrochloride was redetermined, and was found identical with that of the synthetic product in the proximity of $[\alpha]_D^{\circ} = +57$ to $93°$. Many samples crystallized under different conditions were tested, always with the same result. This was a surprising and puzzling

finding; and although there was no doubt in our mind as to the correctness of the early measurement, since all readings in this laboratory are taken by two observers, yet to eliminate all possible doubt the rotation of the original material was again measured and was found as originally recorded $[\alpha]_D^\circ = +129.0$ to 95.0°. It is interesting to note that in the early work of Levene and La Forge the first form occurred on three occasions whereas in subsequent work it could not be obtained again. It is also worthy of note that the value of the molecular rotation of the end carbon atom of the two amino sugars calculated according to Hudson's formula is in good agreement with the value calculated by Hudson for the end carbon atom of other hexoses.

Chondrosaminic Acid.—The observations on chondrosaminic acid and on the acid obtained synthetically originally seemed puzzling and confusing. By the action of hydrocyanic acid on arabinosimine, Fischer and Leuchs[2] obtained pure glucosaminic acid. Because of this it was thought that lyxohexosaminic acid obtained by the action of hydrocyanic acid on lyxosimine was also a uniform substance. The synthetic acid originally obtained in this manner had $[\alpha]_D^\circ = -3.58$ to $-20.7°$. which differed from chondrosaminic acid with $[\alpha]_D^\circ = -16.15$ to $-29.2°$. On the other hand, the reduction of the synthetic acid led to the same chondrosamine, which on oxidation gave rise to chondrosaminic acid. The confusion, however, was cleared up when it was found that the synthetic sugar on oxidation formed the same amino acid as the natural sugar.

Parallel measurements of the optical rotation were made on samples of the acids from the natural and synthetic sugars and were found identical, $[\alpha]_D^\circ = -17.94$ to $-31.89°$. In the light of this, lyxohexosaminic acid has to be regarded as a mixture of the epimeric acids. Indeed, the material prepared this year had $[\alpha]_D^\circ = -1.85$ to $-8.78°$., which differed from the $[\alpha]_D$ of the original synthetic acid. Obviously under varying conditions of experiment different proportions of the epimeric acids are formed.

α, α_1-Anhydromucic Acid, and α, α_1-Anhydrotalomucic Acids.—Inactive anhydrotetrahydroxyadipic acids were obtained from chondrosamine and from the synthetic lyxohexosaminic acids.

[2] Fischer, E., and Leuchs, H., *Ber. chem. Ges.*, 1903, xxxvi, 24.

So long as the latter was considered an individual substance different from chondrosaminic acid, the observation needed special interpretation. Since, however, it was demonstrated that the synthetic acid was a mixture of two epimers, one being chondrosaminic acid, the puzzling element of the observation was eliminated, the identity of the two anhydrotetrahydroxyadipic acids became evident, and their structure could be no other than that of anhydromucic acid.

The structure of the optically active tetrahydroxyadipic acids is manifested from the fact that the natural sugar gives rise to the acid with exactly the same properties as that derived from the synthetic sugar. Since the structure of the latter is obvious from its origin the structure of the former as anhydrotalomucic becomes certain.

α,α_1-Anhydrotalonic and α,α_1-Anhydrogalactonic Acids.—Since the structure of the α, α_1-anhydrotalomucic and α, α_1-anhydromucic acid has been established it became an easy task to prove the structure of the two monocarboxylic acids. The anhydrotalonic should be convertible into anhydrotalomucic acid, and anhydrogalactonic into anhydromucic acid. The brucine salts of two acids were previously obtained, one with a melting point of 218° and $[\alpha]_D^{20} = -12.4$, and the other with a melting point of 244° and $[\alpha]_D^{20} = -9.40$. It was then shown that the former on oxidation gives anhydrotalomucic while the latter forms anhydromucic acid. The former was assumed to possess the structure of anhydrotalonic, the latter of anhydrogalactonic acid. The assumption was fully justified and stands correct in the light of the new evidence.

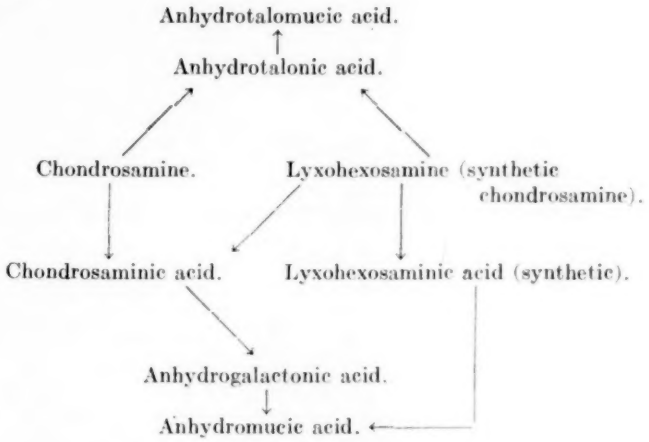
It may be mentioned in this place that formerly the formation of anhydrotalonic acid from the synthetic lyxohexosaminic had seemed puzzling. In view of the fact that lyxohexosaminic acid is recognized as a mixture of two epimers, the source of the anhydrotalonic is to be looked for in one of the two epimers, namely, in epichondrosaminic acid.

Pentacetyl Derivatives.

As a final point proving the identity of the two sugars may be mentioned the fact that they yield the identical pentacetates. Both the α and the β forms obtained by Hudson and Dale from the

natural product were then obtained from the synthetic sugar. It may be noted in this place that Hudson and Dale designated the more soluble pentacetate of chondrosamine with $[\alpha]_D^{20} = 101.3°$ as the β form and the more insoluble with $[\alpha]_D^{20} = 11.0°$ as the α form. This nomenclature was perfectly justified when it was assumed that chondrosamine was *l*-ribohexosamine. Inasmuch as chondrosamine is now recognized as *d*-lyxohexosamine, the form with the higher rotation has to be named the α form, $[\alpha]_D^{20} = 101.3°$, and the one with $[\alpha]_D^{20} = +11.0$, the β form.

The mutual relationship of the derivatives of chondrosamine on one hand and of the derivatives and of the parent substances of the synthetic sugar on the other is represented in the following diagram.



EXPERIMENTAL.

Preparation of Synthetic Chondrosamine Hydrochloride.—The process was essentially the same as previously described.[3] A slight improvement was introduced in that all solutions were concentrated at a temperature of the water bath not exceeding 40°C. It was found convenient to recrystallize the sugar by dissolving it in a minimum amount of water and adding ethyl alcohol saturated with hydrochloric acid. About 35.0 gm. of the synthetic

[3] Levene, P. A., *J. Biol. Chem.*, 1916, xxvi, 143, 155.

sugars were prepared. The specific rotation of the substance was the following.

$$[\alpha]_D^0 = \frac{+1.50 \times 2.0392}{1 \times 0.0516} = +59.30° \quad \text{Initial.}$$

$$[\alpha]_D^{25} = \frac{+2.50 \times 2.0392}{1 \times 0.0516} = +98.80° \quad \text{Equilibrium.}$$

α and β Forms of Chondrosamine Hydrochloride.—A sample of chondrosamine recrystallized out of water and ethyl alcohol saturated with hydrochloric acid.

$$[\alpha]_D^0 = \frac{+1.54 \times 2.0385}{1 \times 0.0516} = +53.14 \quad \text{Initial.}$$

$$[\alpha]_D^{25} = \frac{+2.28 \times 2.0392}{1 \times 0.0514} = +90.42 \quad \text{Equilibrium.}$$

A sample was dissolved in a minimum amount of water, glacial acetic acid was added until the substance began to crystallize, the mixture brought to a boil, and filtered. A solution was made of exactly 2.5 cc. volume.

$$[\alpha]_D^0 = \frac{+3.04 \times 2.5}{1 \times 0.125} = +60.4° \quad \text{Initial.}$$

$$[\alpha]_D^{25} = \frac{4.5 \times 2.5}{1 \times 0.125} = +90.0° \quad \text{Equilibrium.}$$

A sample crystallized out of a minimum amount of aqueous hydrochloric acid.

$$[\alpha]_D^0 = \frac{+2.60 \times 2.5}{1 \times 0.1272} = +57.10° \quad \text{Initial.}$$

$$[\alpha]_D^{25} = \frac{+4.80 \times 2.5}{1 \times 0.1272} = +94.20° \quad \text{Equilibrium.}$$

No attempt was made to explain the slight discrepancies in the solutions since the principal object was to determine conditions controlling the formation of either one of the forms. As all attempts to obtain a sample with the original rotation failed, the rotation of the original material was redetermined. Dr. J. López-Suárez and Dr. G. M. Meyer controlled the reading.

$$[\alpha]_D^0 = \frac{+2.50 \times 2.5}{1 \times 0.0500} = +129.0° \quad \text{Initial.}$$

$$[\alpha]_D^{25} = \frac{+1.90 \times 2.5}{1 \times 0.500} = +96.0° \quad \text{Equilibrium.}$$

Calculating on the basis of Hudson's formula, the molecular rotation of the end carbon atom $= \frac{(129.0 - 51.0)}{2} 215.5 = 8{,}400.$ The value found by Hudson for hexoses was in the neighborhood

of 8,000. The original form is to be regarded as the α and the new as the β form.

Chondrosaminic Acid from Natural and Synthetic Sugars.—This acid was obtained from both the natural and synthetic sugar by oxidation with mercuric oxide. The conditions given by Pringsheim and Ruschmann[4] for oxidation of glucosamine had to be modified.

4.0 gm. of the synthetic sugar were dissolved in 62.0 cc. of water, to the solution 20.0 gm. of mercuric oxide were added, and the mixture was warmed on the water bath for 6 minutes. The reaction product was filtered immediately, the filtrate was freed from mercury by means of hydrogen sulfide, the filtrate from the sulfide concentrated to a small volume, under diminished pressure, when the acid crystallized in the distilling flask. The substance was recrystallized once. It did not melt, but turned light brown at 190°, the same as the acid obtained from the natural sugar. A parallel measurement of the rotation of each product gave the following results.

Natural product.

Initial in 2.5 per cent HCl.

$$[\alpha]_D^0 = \frac{-0.90 \times 2.5}{1 \times 0.1254} = -17.94°$$

Equilibrium.

$$[\alpha]_D^{25} = \frac{-1.60 \times 2.5}{1 \times 0.1254} = -31.89°$$

Synthetic.

Initial in 2.5 per cent HCl.

$$[\alpha]_D^0 = \frac{-0.94 \times 2.5}{1 \times 0.1256} = -17.94°$$

Equilibrium.

$$[\alpha]_D^{25} = \frac{-1.60 \times 2.5}{1 \times 0.1256} = -31.87°$$

Lyxohexosaminic acid.

$$[\alpha]_D^0 = \frac{+0.37 \times 15.0}{2.0 \times 1.496} = +1.85°$$

$$[\alpha]_D^{25} = \frac{-1.73 \times 15.0}{2.0 \times 1.4946} = -8.75°$$

The analysis of the synthetic acid gave the following results.

0.1050 gm. of substance gave 0.1426 gm. CO_2 and 0.0618 gm. H_2O.

	Calculated for $C_6H_{13}NO_5$:	Found:
C	36.92	37.03
H	6.66	6.58

Thus the identity of the two chondrosaminic acids is established.

[4] Pringsheim, H., and Ruschmann, G., *Ber. chem. Ges.*, 1915, xlviii, 680.

Anhydrotalonic Acid.—The acid was previously obtained in small quantities as a brucine salt. The brucine salt was prepared in larger quantities from the natural sugar and from the sugar obtained from lyxohexosaminic acid. Lots of 30.0 gm. of chondrosamine hydrochloride were dissolved in 150.0 cc. of water. To the solution 30.0 gm. of silver nitrite and a few drops of hydrochloric acid were added. The mixture was allowed to react for 6 hours. The silver chloride was then removed by filtration, the filtrate was placed on a boiling water bath for 5 minutes, then treated with a slight excess of hydrochloric acid, and again filtered. To the filtrate 65.0 gm. of bromine were added and the mixture was allowed to stand at room temperature for 3 days. The remaining traces of bromine were removed by shaking the solution with mercury, and the hydrobromic acid by means of lead carbonate and silver carbonate. Finally the nitric acid still present in the solution was removed by the aluminum amalgam method. The resulting solution was transformed into the brucine salt in the usual manner. The solution of the brucine salt was concentrated to a small volume when, on cooling, the brucine salt was crystallized out. When first obtained the substance was readily soluble in methyl alcohol. However, on repeated recrystallization its solubility diminished, so that towards the end it dissolved only in a large volume of boiling methyl alcohol. The salt then crystallized in large polygonal prisms. The melting point was 218°C. and the optical rotation was the following.

$$[\alpha]_D^{20} = \frac{-0.63^\circ \times 10.0}{2 \times 2.522} = -12.4^\circ$$

The analysis of the substance gave the following results.

0.1063 gm. of substance on drying under diminished pressure at the temperature of xylene vapors lost 0.003 gm. of water.

	Calculated for $C_{29}H_{31}N_2O_{19}+H_2O$:	Found:
H_2O	3.11	3.58

0.1025 gm. of the dry substance gave 0.2284 gm. CO_2 and 0.0569 gm. H_2O.

	Calculated for $C_{29}H_{30}N_2O_{19}$:	Found:
C	60.80	60.78
H	6.10	6.15

From the synthetic lyxohexosaminic acid the substance was prepared in the following manner. 30 gm. of the acid were dissolved in a solution of 200.00 cc. of water and 40.0 cc. of 10 per cent hydrochloric acid. 40.0 gm. of silver nitrite were added. The following morning 10.0 gm. of silver nitrite and 10.0 cc. of 10 per cent hydrochloric acid were added. After 30 hours from the beginning of the experiment, the silver chloride was removed by filtration and other remaining silver by hydrogen sulfide. From the filtrate the nitric and nitrous acids were removed by the aluminum amalgam method.

It had the following composition.

0.1012 gm. of substance on drying in a xylene bath under diminished pressure lost 0.0038 gm. of H_2O.

0.0974 gm. of substance, dried, on combustion gave 0.2158 gm. CO_2 and 0.0562 gm. H_2O.

	Calculated for $C_{29}H_{48}N_2O_{16}H_2O$:	Found:
H_2O	3.11	3.75

	Calculated for $C_{29}H_{48}N_2O_{16}$:	Found:
C	60.80	60.42
H	6.10	6.45

The substance had a melting point of 218°C. and the following rotation.

$$[\alpha]_D^{20} = \frac{-0.655^\circ \times 10}{2 \times 2.66} = -12.3$$

Thus the identity of the acid obtained from natural chondrosamine and of the one obtained synthetically was established. In order to prove its identity with α, δ-anhydrotalonic acid it was oxidized by means of nitric acid. 21.0 gm. of the brucine salt were freed from brucine by means of barium hydroxide and chloroform. After the removal of barium, the solution was concentrated to 40.0 cc., an equal volume of concentrated nitric acid was added, and the solution was boiled over a free flame until a lively evolution of red fumes set in. The solution was then transferred to a large clock glass and evaporated to dryness. The residue was dissolved in water and again evaporated to dryness. This operation was repeated. The reaction product was converted into the calcium salt. The salt was recrystal-

lized twice. Each time the calcium salt was decomposed by a little less than the calculated amount of oxalic acid, and then reconverted into the calcium salt.

0.1008 gm. of the air-dry substance on drying in a xylene bath under diminished pressure lost 0.0130 gm. of H_2O.

	Calculated for $C_6H_8O_8Ca+3H_2O$:	Found:
H_2O	12.68	12.90

0.0878 gm. of dry substance gave on combustion 0.0936 gm. CO_2 and 0.0274 gm. H_2O.

0.0896 gm. of the substance gave 0.0206 gm. CaO.

	Calculated for $C_6H_8O_7Ca+H_2O$:	Found;
C	29.03	29.07
H	3.22	3.49
Ca	22.58	22.99

The optical rotation of the substance in a 10 per cent solution of HCl was the following.

$$[\alpha]_D^{20} = \frac{-0.30^\circ \times 2.50}{1 \times 0.100} = -7.5^\circ$$

Thus the anhydrohexonic acid obtained from lyxohexosaminic acid yields on further oxidation an optically active anhydrotetrahydroxyadipic acid; hence it possesses the structure of anhydrotalonic acid.

Anhydrogalactonic Acid.—10 gm. lots of chondrosaminic acid were treated in the same manner as lyxohexosaminic acid in the above experiment. The brucine salt obtained in this manner melted at 244°, and had the following optical rotation.

$$[\alpha]_D^{20} = \frac{-0.47^\circ \times 10.0}{2 \times 2.508} = -9.37^\circ$$

The composition of the substance was the following.

0.0988 gm. of the substance, dried in a xylene bath, gave 0.2196 gm. CO_2 and 0.0576 gm. H_2O.

	Calculated for $C_{29}H_{30}N_2O_{10}$:	Found:
C	60.80	60.73
H	6.10	6.53

10.0 gm. of the brucine salt were freed from brucine as in the above experiment. The brucine-free solution was evaporated

to 25 cc. and then diluted with an equal volume of concentrated nitric acid and boiled over free flame until the volume was reduced to about 20.0 cc., then 10.0 cc. of nitric acid were again added, and the solution was again boiled, over free flame. When the solution was concentrated to 20.0 cc. it was transferred to a clock glass and concentrated on a boiling water bath to dryness. The substance immediately crystallized. It was redissolved in water and again evaporated. The operation was repeated once more. The final crystalline residue was dissolved in hot acetone and very little ether was added, when a small amorphous precipitate formed. This was removed by filtration, and the filtrate was allowed to crystallize. The crystals were filtered, redissolved in hot acetone, and allowed to crystallize. The final product melted at 205°C.

0.1600 gm. of the substance dissolved in 2.5 cc. showed no optical activity.

On the basis of this the identity with anhydrogalactonic acid of the monohydroxy acid obtained from chondrosaminic acid is established.

α,α₁-Anhydrotalomucic Acid from the Synthetic Sugar.—60 gm. of the synthetic lyxohexosamine hydrochloride were dissolved in 30.0 cc. of water containing 1 cc. of hydrochloric acid. 8.0 gm. of silver nitrate were added and the mixture was allowed to stand for 6 hours. It was then filtered. The silver was removed from the filtrate by means of hydrogen sulfide. The clear filtrate from the silver sulfide was concentrated to 20.0 cc., cooled to 0°C., and diluted with 20.0 cc. of nitric acid also cooled at 0°C. The solution was allowed to stand over night. It was then boiled over a free flame until a lively evolution of yellow fumes set in. The solution was then transferred to a clock glass, evaporated on a water bath, and the calcium salt was then prepared in the usual way. The salt was reprecipitated twice and had the following composition.

0.1054 gm. of substance on drying in a xylene bath lost 0.0130 gm. H_2O.

	Calculated for $C_6H_9O_5Ca+2H_2O$:	Found:
H_2O	12.68	12.33

0.0924 gm. of the dry substance gave on combustion 0.0982 gm. CO_2 and 0.0298 gm. H_2O and 0.0924 gm. CaO.

	Calculated for $C_6H_8O_7Ca+H_2O$:	Found:
C	29.03	28.98
H	3.22	3.60
Ca	22.58	23.05

The optical rotation of the substance is the following.

Initial. $[\alpha]_D^{20} = \frac{-0.32° \times 2.5}{1 \times 0.100} = -8.0°$ Equilibrium. $[\alpha]_D^{20} = \frac{-0.32° \times 2.5}{1 \times 0.100} = -8.0°$

in 10 per cent HCl.

Hence the substance is α,α_1-anhydrotalomucic acid. It is identical with the substance previously obtained from natural chondrosamine.

Pentacetyl Derivative of the Synthetic Chondrosamine.—The substance was prepared from the natural chondrosamine by Hudson and Dale.[5] Practically the same conditions were followed in this work.

In 30.0 cc. of acetic anhydride 5.0 gm. of zinc chloride were dissolved. To this solution 5.0 gm. of the hydrochloride were added and the mixture was warmed gently until a lively reaction developed. The reaction was kept up for 2 minutes, then the reaction product was poured into 100 cc. of water cooled to 0°C. The mixture was neutralized with potassium bicarbonate, transferred to a separatory funnel, and extracted with chloroform. The chloroform extract was washed with water. Over the chloroform a layer of crystals appeared which were insoluble in water. The crystals consisted of the more insoluble fraction of the pentacetates. The chloroform extract was evaporated to dryness *in vacuo*, the residue was recrystallized out of alcohol, and from the mother liquor a second crop of crystals was obtained. The top fraction consisted of the pure α form while the most soluble form was practically the pure β form.

The α form turned slightly brown at 232° and melted with decomposition at 237°C. (corrected). Its optical rotation in chloroform solution was the following.

[5] Hudson, C. S., and Dale, J. K., *J. Am. Chem. Soc.*, 1916, xxxviii, 1431.

$$[\alpha]_D^{20} = \frac{+ 0.07 \times 20.0}{2 \times 0.0802} = + 8.75°$$

A similar fraction from natural chondrosamine had a melting point of 235°C. and the following optical rotation.

$$[\alpha]_D^{20} = \frac{0.09 \times 20.0}{2 \times 0.075} = + 12.0°$$

Hudson and Dale found for their β form a melting point of 235° (with decomposition) $[\alpha]_D = + 11.00°$.

The β form was apparently not quite pure, but taking into consideration the small quantity of starting material it is rather surprising that each form could be separated with so little difficulty.

The melting point of the β form was very sharp at 197°C. and the optical rotation in chloroform solution was

$$[\alpha]_D^{20} = \frac{+ 0.90 \times 20.0}{2 \times 1.000} = + 90.0°$$

Hudson and Dale found for the α form the melting point 182–183°C. and $[\alpha]_D$ 101.3°.

The composition of the pentacetyl derivative was the following.

0.1014 gm. of the substance gave 0.1834 gm. CO_2 and 0.0560 gm. H_2O.

	Calculated for $C_6H_8NO_5(CH_3CO)_5$:	Found:
C	49.49	49.32
H	5.96	6.18

THE RELATION BETWEEN THE CONFIGURATION AND ROTATION OF EPIMERIC MONOCARBOXYLIC SUGAR ACIDS.

III. THE PHENYLHYDRAZIDES.

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In previous publications[1] it was demonstrated that the direction of the optical rotation of the α carbon atom of a pair of epimeric monocarboxylic sugar acids is determined by its configuration. When the hydroxyl of the α carbon atom is in the same position as in *d*-gluconic acid (on the right) the direction of the rotation is right, while when it is in the same position as in *d*-mannonic acid the direction of the rotation is left. The rule was found valid for phenylhydrazides, brucine, strychnine, and sodium and calcium salts.

In the early phase of the work it seemed that in salts of different acids with the same base and possibly in all derivatives of the sugar acids, the value of the molecular rotation of the α carbon atom was constant. If that were true the magnitude of the rotation of the α carbon atom of a given salt would serve as an index of its purity. Following our observation, Hudson[2] succeeded in demonstrating that in phenylhydrazides the magnitude of the rotation of the β, γ, and δ carbon atoms of several monocarboxylic acids were insignificant as compared with that of the α carbon atom, so that the direction of the rotation of the phenylhydrazide could be determined by that of the α carbon atom. Because of this Hudson suggested that, *vice versa*, the configuration of the α carbon atom in a given sugar acid may be

[1] Levene, P. A., *J. Biol. Chem.*, 1915, xxiii, 145. Levene, P. A., and Meyer, G. M., *ibid.*, 1916, xxvi, 355.

[2] Hudson, C. S., *J. Am. Chem. Soc.*, 1917, xxxix, 462.

determined by the direction of the rotation of its phenylhydrazide. Wherever the phenylhydrazide is obtainable this undoubtedly is correct. In our first note on this subject only one pair of epimeric phenylhydrazides was discussed, and further work was in progress when Hudson's article appeared. We did not discontinue our work for the reason that it has furnished some interesting results regarding the numerical value of the α carbon atom in different derivatives of the same acid.

As already mentioned, it was expected that the molecular rotation of the α carbon atom would be constant in all compounds of such acids, the purity of which could not be questioned. A scrutiny of the empirical values for the molecular rotation of the α carbon atom for the various pairs of epimeric salts revealed considerable variations, and it remained uncertain whether these were occasioned by the impurity of some of the acids.

The maximum rotation of the α carbon atom of a pair of acids is obtained when both acids are perfectly free from the epimeric acid, since the rotation of the α carbon atom of each of two epimers is in opposite directions. There can scarcely be any doubt that derivatives of gluconic and mannonic acids can be obtained in perfect purity, since both acids form crystalline lactones. Hence it was surprising to find that the rotation of the α carbon atom in the pair of allonic and altronic hydrazides was of a higher value than that of the gluconic-mannonic pair. On the other hand, in the gulonic-idonic pair the magnitude of rotation was equal to that of the gluconic-mannonic pair. Hence it is justifiable to accept the purity of idonic acid. On the other hand, the value of the rotation of the α carbon atom in the talonic-galactonic pair was much lower than that of any other pair. Besides, the rotation of both hydrazides was found to be in the same direction. Since galactonic lactone is obtained in a beautifully crystalline form, there exists no doubt as to its purity. On the contrary, the hydrazide of talonic acid was obtained from the brucine salt, and there always existed among workers a certain scepticism as to the purity of this compound. The brucine salt employed in this work has the rotation of $[\alpha]_D^{20} = -26.15°$. This is the highest value recorded for the rotation of the brucine salt of talonic acid.

The scrutiny of the data obtained in the course of this work indicates that the magnitude of the rotation of the α carbon atom is not altogether constant even for the series of phenylhydrazides, but the rule of the relation of direction of the rotation to the configuration of the α carbon atom remains valid.

In the present work all readings were made on two solutions prepared from the same sample so as to reduce to a minimum the experimental error.

Phenylhydrazide.	Author.	C	T°.	[α]	$\frac{A+B}{2}$
Gluconic.		1	85	+18.0	
		1	85	+18.2	
	Nef.[3]	2	20	+12.0	
Mannonic.		1	85	−10.5	14.25°
		1	90	−10.7	
	Hudson.[2]	2	80	− 8.1	
Gulonic.		1	20	+13.45	
		1	20	+13.87	
	Nef.	4	20	+13.74	
Idonic.		1	20	−15.1	14.25°
		1	20	−15.3	
	Nef.	2	20	−12.42	
Galactonic.		1	25	+12.2	
	Nef.	2	20	+10.44	8.25°
Talonic.		0.5	25	+ 4.35	
Allonic.		1	20	+25.88	
		1	20	+25.50	
Altronic.		1	20	−15.8	20.8°
		1	20	−15.9	
Arabonic.		2.5	63	−16.09	
	Hudson.	1	20	−14.5	16.09°
..ibonic.		2.5	63	+16.09	

[3] Nef, J. U., *Ann. Chem.*, 1914, cdiii, 204.

EXPERIMENTAL.

The phenylhydrazides of gluconic, mannonic, galactonic, allonic, and arabonic acids were prepared in the usual way and were recrystallized out of water. They were obtained in the form of perfectly colorless bright plates.

The idonic, gulonic, talonic, altronic, and ribonic acids were precipitated out of alcoholic solutions by ether. A slightly gelatinous perfectly white substance settled out, which crystallized out of alcohol in the form of colorless plates. Idonic acid was prepared by the method of Van Ekenstein and Blanksma.[4]

With a few exceptions all readings were made in a 2 dm. tube. Practically all readings were made with an accuracy of $\pm 0.00°$.

[4] Van Ekenstein, W. A., and Blanksma, J. J., *Rec. trav. chim. Pays-Bas*, 1908, xxvii, 1.

CEREBROSIDES.

III. CONDITIONS FOR HYDROLYSIS OF CEREBROSIDES.

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The conditions of hydrolysis of cerebrosides followed by most investigators remain as they were introduced by Thudichum,[1] the pioneer worker in this field. Thudichum made use of two methods. One consisted in heating the cerebrosides in a 2 per cent aqueous sulfuric acid solution in sealed tubes for a period varying from 310 to 370 hours. In the second method, the substance was decomposed by heating a solution in ethyl alcohol containing sulfuric acid, under a reflux. These methods remain quite serviceable for experiments of a qualitative nature. However, the methods proved imperfect for an investigation into the quantitative relationships of the components of cerebrosides. Indeed, Thudichum never intended them for that purpose. On the other hand subsequent workers who attempted the quantitative analysis of cerebrosides followed Thudichum's directions without having ascertained the degree of their accuracy.

Early in our work on the hydrolysis of cerebrosides we became aware of the fact that the original methods of hydrolysis demanded improvement from the standpoint of convenience and reliability. The original aqueous hydrolysis was carried out for a period of over 300 hours. It is evident that this prolonged heating must bring about a partial decomposition of the galactose. Again, the alcoholic hydrolysis (partial alcoholysis) was found both inconvenient and inaccurate. Thudichum had demonstrated the presence of some intermediate substances among the products of hydrolysis. Furthermore, the mineral acid in the

[1] Thudichum, J. L. W., Physiological Chemistry of the Brain, London, 1884.

presence of alcohol catalyzes not only the process of splitting the original substances, but also the synthetic processes between alcohol and the cleavage products. Hence the products of reaction on boiling the cerebrosides with an alcoholic solution of a mineral acid are galactose, ethyl galactoside, fatty acids and their ethyl esters, sphingosine, and mono- and diethyl sphingosine. The separation of these substances is not a simple matter.

Because of this, an attempt was made to find conditions of hydrolysis under which the components of the cerebrosides could be obtained in a fair degree of purity and in a quantity approaching the values required by theory. It was found that two operations were required, one for determining the sugar content, and a second for determining the base and acid. In connection with the sugar estimation the older methods particularly needed improvement. During a long period when the older methods of hydrolysis were followed in our laboratory, a yield of glucose approaching the theoretical requirement was obtained only once. A scrutiny of the results published by Thierfelder[2] and his coworkers shows that they also encountered in individual samples of cerebrosides greater sugar variations than one should have expected from the practically constant elementary composition of the substances.

Hence we undertook to determine the destructive influence on galactose of mineral acids in concentrations usually employed for hydrolysis of cerebrosides. The details are given in the tables.

On the basis of these tables the following conditions were selected for the hydrolysis of cerebrosides, aiming at the sugar estimation. 1.0 gm. of cerebrosides and 16 cc. of 3 per cent sulfuric acid were heated with shaking in a sealed tube for 12 hours at 105°. The filtrate from the insoluble cake combined with the wash water was used for the sugar estimation. When larger quantities are employed for hydrolysis the proportions remain the same. Under such conditions approximately 90 per cent of the sugar present in the cerebroside is obtained. We were unable to obtain more accurate results. We also wish to remark that when a small quantity of cerebroside is used the sugar

[2] Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, 1911, lxxiv, 282. Thierfelder, *ibid.*, 1913, lxxxv, 35; 1914, lxxxix, 236. See also Argiris, A., *ibid.*, 1908, lvii, 289.

TABLE I.

Galactose.

I. 3 gm. of galactose dissolved in 100 cc. of 10 per cent HCl + C_2H_5OH, 4:1. Portions of 20 or 10 cc. hydrolyzed for various lengths of time.

	Per cent.	Loss.	Per cent loss.
Original solution	0.546	0	0
8 hrs.	0.380	0.166	30.40
12 "	0.304	0.242	44.40
24 "	0.131	0.415	76.00
48 "	0.022	0.524	96.00

II. 3 gm. of galactose dissolved in 100 cc. of 2 per cent HCl.

	Per cent.	Loss.	Per cent loss.
Original solution	0.511	0	0
2 hrs.	0.511	0	0
4 "	0.4965	0.0145	2.85
8 "	0.4825	0.0285	5.54
24 "	0.4590	0.0520	10.23

III. 3 gm. of galactose dissolved in 100 cc. of 4 per cent H_2SO_4.

	Per cent.	Loss.	Per cent loss.
Original solution	0.5355	0	0
2 hrs.	0.5355	0	0
4 "	0.5105	0.0250	4.66
8 "	0.5070	0.0285	5.34
24 "	0.4820	0.0535	10.00

IV. 1 gm. of galactose and 40 cc. of alcohol containing 7 per cent H_2SO_4 boiled for 4 hours and then hydrolyzed with 3 per cent H_2SO_4 for various lengths of time.

	Sugar.
	gm.
Solution before hydrolysis	0.0000
1 hr. hydrolysis	0.6000
3 " "	0.8925
4 " "	0.6950

TABLE I—*Concluded.*

Phrenosin.

I. 2 gm. of phrenosin boiled with 40 cc. of 10 per cent HCl + 95 per cent alcohol 4: 1 for varying lengths of time.

Amount of phrenosin.	Time of boiling.	Sugar.	
gm.	*hrs.*	*gm.*	*per cent*
2.0000	8	0.205	10.25
1.9946	12	0.084	0.42
1.9974	24	0.031	0.15
2.0004	48	0.000	0.00

II. 3 gm. of phrenosin boiled with absolute alcohol and 40 cc. of 7 per cent H_2SO_4 for 4 hours, and then hydrolyzed with 4.4 per cent H_2SO_4.
Found.....11.8 per cent.

III. 3 gm. of phrenosin hydrolyzed with 50 cc. of 3 per cent H_2SO_4 + 5 cc. of alcohol.

	Sugar
hrs.	*per cent*
16	17.2
24	16.3

IV. 1 gm. of phrenosin heated in a sealed tube with 16 cc. of 3 per cent H_2SO_4 at 100° for various lengths of time.

	Sugar.
hrs.	*per cent*
2	6.8
4	15.48
8	16.32
11	17.98
14	18.25
16	17.68
24	16.60

Analyzed according to Rosenheim's method (*Biochem. J.*, 1916, x, 146), 11.56 per cent.

cannot be estimated polarimetrically with a sufficient degree of accuracy.

TABLE II.

Hydrolysis in Sealed Tube for 12 Hours at 105°.

Sample No.	Sugar.
	per cent
60 C	18.8
61 C	18.6
62 C	21.0
272 C	19.2
366 C	18.8
514 C	18.72
Theory.	21.8

For the purpose of estimating the base and the fatty acids the following conditions were found satisfactory: 1 gm. of cerebroside was heated with 3 per cent sulfuric acid in a sealed tube with shaking for 24 hours at 105°. After cooling the reaction product was filtered. The insoluble cake was washed with distilled water on the filter, then pressed between filter paper to remove adhering water. The filter paper with the substance was transferred to boiling methyl alcohol to which a few drops of phenolphthalein were added. This was followed by the addition of a hot saturated solution of barium hydroxide in methyl alcohol until the reaction was strongly alkaline. Acetone was then added as long as a precipitate formed. When all the barium hydroxide and the barium salts are precipitated the pink solution turns colorless. The solution contains the base, and the precipitate consists of the barium salts of the fatty acids. For the purpose of purification the fatty acids were liberated with hydrochloric acid, and redissolved with acetone. This operation was repeated three times. The soaps were suspended in water, decomposed with hydrochloric acid, a little benzene was added, and the flask was placed on a water bath until the benzene was evaporated. On cooling, the acids floated on the surface of the liquid. They were then filtered and dissolved in hot acetone to remove the inorganic salts. The acetone solution on evaporation left a dry residue, ready for analysis. All the mother liquors were added to the original solution containing the base. The combined solu-

tion was evaporated nearly to dryness and extracted with hot acetone. The operation was repeated until the final residue was completely soluble in acetone. All the insoluble residues were added to the soaps. The acetone solution of the bases was evaporated and dried to constant weight.

This method has been tried out for 4 years and has always given results which were as accurate as could be expected for substances of that nature. Table III contains the results of only a few of the analyses carried out by this method.

TABLE III.

Sample No.	Base.	Fatty acid.
	per cent	*per cent*
60 C	32.7	43.0
61 C	31.0	48.6
62 C	30.0	42.8
366 C	35.8	48.52
514 C	32.1	46.7
Theory.	34.5	48.1

The results of the elementary composition of the individual fractions were as follows.

The acids were analyzed directly after removing the acetone, if they were free from inorganic impurities. Otherwise, they were dissolved in ether and freed from impurities by shaking with dilute hydrochloric acid and then with water. The ethereal solution was then freed from ether and the residue analyzed.

60 C 0.1000 gm. substance gave 0.2890 gm. CO_2 and 0.1150 gm. H_2O.
61 C 0.1040 " " " 0.2888 " " " 0.1162 " "
62 C 0.1050 " " " 0.2894 " " " 0.1162 " "
366 C 0.1055 " " " 0.2886 " " " 0.1142 " "
514 C 0.1070 " " " 0.3018 " " " 0.1200 " "

	Calculated for:		Found:				
	$C_{24}H_{48}O_2$:	$C_{28}H_{59}O_3$:	60 C	61 C	62 C	366 C	514 C
C.....	78.2	75.33	75.10	75.74	75.16	74.60	77.00
H.....	13.2	12.50	12.25	12.50	12.38	12.10	13.03

The base was analyzed in form of the sulfate. In order to obtain this, the fraction was dissolved in alcohol, and an alcoholic solution of sulfuric acid was added until the mixture reacted markedly acid to litmus. The mixture was allowed to stand 15 to 24 hours at 0°C., filtered, and washed, first with acetone and then with ether, and dried under diminished pressure.

60 C 0.1064 gm. substance gave 0.2226 gm. CO_2 and 0.0962 gm. H_2O.
61 C 0.0998 " " " 0.2334 " " " 0.0998 " "
62 C 0.0984 " " " 0.2264 " " " 0.0976 " "
514 C 0.1012 " " " 0.2252 " " " 0.0980 " "

	Calculated for $(C_{17}H_{33}NO_2)_2\ H_2SO_4$:	Found:			
		60 C	61 C	62 C	514 C
C.....	61.08	60.76	64.42	62.87	61.29
H.....	10.78	10.00	11.28	11.12	10.95

The analytical figures for the sulfates deviate considerably in some instances from the theory. This however, is not due to the presence of impurities but occurred when an insufficient amount of sulfuric acid was added to form the neutral salt. One or two recrystallizations generally suffice to obtain a pure product. With more experience in the work analytically pure samples are obtainable without recrystallization.

50 gm. of material are a convenient quantity for analysis. Very satisfactory results were obtained when 1.5 gm. were hydrolyzed. When the analysis of the fatty acid fraction obtained by this method shows the presence of both lignoceric and cerebronic acid, and when the identification of each one is desired it is advantageous to resort to a separate hydrolysis for each acid. For the isolation of cerebronic acid, the procedure is as follows.

10 gm. of the cerebrosides and 150 cc. of a 10 per cent hydrochloric acid solution to which 30 cc. of 98 per cent ethyl alcohol are added, are heated for 24 hours in a flask provided with a reflux condensor and a mechanical stirrer. On cooling, a solid cake is formed on the surface of the liquid. This is dissolved in hot methyl alcohol. A solution of barium hydroxide is then added and the soaps are purified exactly in the manner described above.

The acid obtained in this manner generally analyzes as follows:

A sample of cerebroside (94 C) with $[\alpha]_D^{20} = 0.00°$:
0.1144 gm. substance gave 0.3176 gm. CO_2 and 0.1257 gm. H_2O.
C = 75.71; H = 12.30.
A sample of cerebroside (221 B) with $[\alpha]_D^{20} = -2.0°$:
0.1166 gm. substance gave 0.3248 gm. CO_2 and 0.1317 gm. H_2O.
C = 75.85; H = 12.64.

From the optical rotation of the substances it is evident that they contained cerasin. On the other hand samples of the same nature yield pure lignoceric acid when decomposed by means of alcohol containing sulfuric acid. The details are as follows.

10.0 gm. of cerebroside are taken up in 100 cc. of 99.5 per cent alcohol containing 10.0 gm. of sulfuric acid and heated in a boiling water bath for 8 hours. The solution is allowed to cool over night at 25°C. Ethyl lignocerate settles out in the form of bright scales. These are removed by filtration. The precipitate may be dissolved in ether, the solution dried over potassium carbonate, filtered, and analyzed in the form of the ester. It also may be converted into the free acid.

Treated in this manner a sample (24 B) with $[\alpha]_D^{20} = 0.00°$ yielded an acid of the following composition:

0.1183 gm. substance gave 0.3388 gm. CO_2 and 0.1139 gm. H_2O.
C = 78.10; H = 13.15.

CEREBROSIDES.

IV. CERASIN.

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Thudichum[1] was the first to assume the existence of more than one cerebroside in brain tissue. By fractionation from alcohol he succeeded in isolating from the crude cerebroside mixture two substances. One he considered an individual glucoside, which he named phrenosin. The other substance he regarded as a second cerebroside, cerasin, still containing some phrenosin. Apparently for lack of material, Thudichum did not accomplish the task of preparing a pure sample of cerasin, nor did he furnish any information regarding the chemical differences of the two cerebrosides.

Subsequent workers have rediscovered phrenosin[2] and, in a general way, have substantiated Thudichum's claim for its individuality.

Regarding the second cerebroside, cerasin, no essential progress was made for many years. The substances, phrenosin and cerasin, were distinguished by their solubilities and their physical appearance. However, in 1913, a chemical distinction between phrenosin and cerasin was twice discovered, nearly simultaneously. It was found that from the so called cerasin, a fatty acid of the composition, $C_{24}H_{48}O_2$, could be isolated, whereas pure phrenosin contained only cerebronic acid, $C_{25}H_{50}O_3$. In this respect it is worthy of note that Thudichum foresaw the possibility of the two cerebrosides differing only by the nature of their

[1] Thudichum, J. L. W., Physiological Chemistry of the Brain, London, 1884.

[2] Loening, H., and Thierfelder, H., *Z. physiol. Chem.*, 1911, lxxiv, 282; 1912, lxxvii, 202. Thierfelder, *Z. physiol. Chem.*, 1913, lxxxv, 35; 1914, lxxxix, 236.

fatty acids. The new acid was recognized independently by Levene[3] and by Rosenheim[4] as lignoceric acid. There still seems, however, to exist a lack of convincing evidence that cerasin has ever been obtained free from phrenosin. It is true, on the basis of a new test applied to the study of cerebrosides, namely, the selenite plate test, Rosenheim claimed to have isolated pure cerasin. However, comparing the optical activity of the various samples obtained by Rosenheim with those prepared by us, we feel certain that our material was of the same degree of purity as that of the previous author. The samples of Rosenheim varied between $[\alpha]_D = -2.50°$ and $-3.71°$, whereas the $[\alpha]_D$ of our samples varied between $-2.25°$ and $-3.45°$.

The first samples of cerasin of that nature were obtained by us in 1913. The results were not published at that time for the reason that we hoped to obtain purer material by means of the older methods of fractional crystallization, or fractional extraction by means of organic solvents. We soon became convinced, however, that the results of the operations were variable, even when the conditions of purification seemed constant. Evidently some unknown factors played a part and since the factors remained unknown, they could not be controlled. Hence the method of fractionation was abandoned and a search was made for such derivatives of phrenosin and cerasin, which would possess properties suitable for the separation of one from the other.

With this aim in view the properties of the acetyl, benzoyl, cinnamoyl, and *p*-nitrobenzoyl derivatives were investigated. Benzoylation was selected as the simplest and best method for the separation of the two compounds. A convenient way of benzoylation was found in the use of pyridine as a solvent, a method first introduced by Einhorn. The saponification of the benzoyl derivative is best accomplished by means of sodium methylate. These are the two essential points of the process, the details of which are given in the experimental part.

It should be noted here that Rosenheim[5] mentioned the possibility of accomplishing the separation of the two cerebrosides

[3] Levene, P. A., *J. Biol. Chem.*, 1913, xv, 359.

[4] Rosenheim, O., *Tr. Int. Cong. Med.*, 1913, ii, 626; *Biochem. J.*, 1916, x, 142.

[5] Rosenheim, *Biochem. J.*, 1914, viii, 110.

through benzoylation. Thierfelder[6] later suggested the use of the acetyl derivatives.

It must, however, be borne in mind that a satisfactory result is obtained only when the starting material contains a moderate proportion of phrenosin; namely, a mixture with $[\alpha]_D = 0.0°$ in pyridine. Hence, the complete process is composed of an initial step consisting of fractional precipitation or fractional extraction, and a second step, consisting of separation by means of benzoylation. As a result of this second step a product is obtained with $[\alpha]_D = -2.50$ to $-3.50°$.

This levorotation is nearly that obtained on previous occasions by Rosenheim in 1916 and by us in 1913. As in 1913, we are convinced now also that this material is not yet free from phrenosin. This view is based on the fact that, on hydrolysis with aqueous sulfuric acid under conditions described by Levene and Meyer,[7] cerasin, with the highest levorotation, yields a mixture of fatty acids containing about 77.0 per cent carbon. Furthermore, on hydrolysis in a solution of 10 per cent hydrochloric acid, containing 15 per cent alcohol, one obtains either pure cerebronic acid, or a mixture of cerebronic and lignoceric acids, whereas, by using alcohol containing 8 to 10 per cent sulfuric acid (by weight), one obtains pure lignoceric acid. Hence, it is evident that one may be led into an error if he bases his conclusion on the latter mode of cleavage (alcoholysis). The find of Rosenheim may be explained by the fact that he employed this method of hydrolysis.

Thus, a complete separation of cerasin from phrenosin has not as yet been accomplished. The present results are reported at this time because of lack of confidence in a speedy solution of the problem. For the present we are engaged in the preparation of large quantities of material with a rotation of approximately $-3.0°$.

In a way the topic discussed here may appear to possess a merely academic interest, since the existence of the two cerebrosides is quite certain. The only alternative hypothesis would be the existence of one simple cerebroside, phrenosin, while cerasin might be a dicerebroside. This view is contradicted by the molecular

[6] Thierfelder, *Z. physiol. Chem.*, 1914, lxxxix, 248.

[7] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, 1917, xxxi, 627.

weight determinations by Kossel and Freytag,[8] who found a mean value of 986, using glacial acetic acid as the solvent, and by Rosenheim,[9] who found a mean of 763, using Barger's microscopic method, based on vapor pressure, with pyridine as the solvent. However, the final and absolute proof of the existence of a substance is its isolation.

EXPERIMENTAL.

Preparation of the Mixture of Cerebrosides.

The crude cerebrosides used in this work were prepared essentially by the method of Rosenheim.[10] The principal feature of this method consists in dissolving the "white matter" (mixture of cerebrosides and sphingomyelin) in pyridine. The difference in the procedure was that the "white matter" was heated in commercial pyridine, until the solution was complete. This solution was then allowed to stand over night at room temperature. The filtrate contained the cerebrosides. This was concentrated to a small volume and poured into alcohol. The precipitate thus obtained was dissolved in hot 90 per cent alcohol, filtered from insoluble oily material, and again allowed to cool. The precipitate was repeatedly extracted with ether. The final insoluble product was composed of the mixed cerebrosides.

Separation into Phrenosin and Cerasin Fractions.

The separation into phrenosin and cerasin fractions was accomplished by means of acetone. Two fractions were obtained, one practically insoluble in boiling acetone, the other soluble. Each of these fractions was a mixture of two cerebrosides. However, the first (insoluble) contained a very high proportion of phrenosin while in the second (soluble) cerasin was predominating.

The procedure used was as follows: 100 gm. of the cerebrosides were dissolved in 50 cc. of boiling 95 per cent ethyl alcohol and to this solution 3 liters of boiling acetone were added. A precipitate formed immediately. This was removed by filtering with suction,

[8] Kossel, A., and Freytag, F., *Z. physiol. Chem.*, 1893, xvii, 445.
[9] Rosenheim, *Biochem. J.*, 1916, x, 153.
[10] Rosenheim, *Biochem. J.*, 1914, viii, 110; 1916, x, 142.

care being taken to warm the funnel before filtration. The insoluble part yields practically pure phrenosin when redissolved in boiling alcohol and then allowed to crystallize in a thermostat at 42°C.

The acetone-solution fraction was concentrated to a small volume and the cerebrosides were allowed to separate out. The material obtained in this manner was further fractionated from methyl alcohol. For this purpose it was dissolved in methyl alcohol and then allowed to stand at 40°. This gave the fraction I a_1. The filtrate from I a_1 was then allowed to cool to room temperature when a second precipitate settled out. This was designated I a_2. On cooling to 0°C. a third fraction, I a_3, was obtained.

The further purification of each of these fractions was laborious, various modifications of Rosenheim's original method being tried, and the results were variable. These fractions have no interest since the introduction of the benzoylation process. In order to obtain material for this process, the crude cerebrosides may be dissolved in a hot solution of one part ethyl alcohol and three parts chloroform (by volume). On standing at room temperature a precipitate forms, which is removed by filtration. The mother liquor is concentrated and either allowed to crystallize or is poured into acetone. Usually this material has a rotation of about 0.0° (in pyridine). If it is higher (+0.02 to +0.04°) the operation is repeated. In some cases it was found that the mother liquor of the crystallization of the crude cerebrosides from ten parts of ethyl alcohol also gave a product with a rotation of $[\alpha]_D = 0.0°$.

Further Separation of Cerasin from Phrenosin by Benzoylation.

As mentioned above, Rosenheim[5] first suggested the possibility of a separation of phrenosin and cerasin by means of the benzoyl derivatives. We have also examined the acetyl derivatives as a method of separation but find it unsatisfactory, especially when compared with the benzoyl method.

The acetyl derivative of the material with a rotation of about −0.02° was prepared as described below and separated into two fractions, soluble and insoluble in methyl alcohol at 0°. The two fractions were then saponified with sodium methylate, and gave products with $[\alpha]_D$ of − 0.02° and − 0.02°. This indicates that no separation has been effected.

The benzoyl derivative was prepared in pyridine as described on page 644. The product, after removing the ether on the water bath, is taken up in methyl alcohol and cooled to 0° over night. The supernatant liquid is decanted, concentrated to a small volume, poured into a solution of sodium methylate in methyl alcohol, and the mixture heated 2 hours. Usually the cerebroside separates out during the heating. After cooling, the precipitate is filtered off, washed thoroughly with methyl alcohol and then with acetone, and crystallized from methyl alcohol until ash-free. It is usually necessary to decolorize the solution with animal charcoal in order to obtain a colorless product. Such material showed values of $[\alpha]_D$ varying from -2.2 to $-3.2°$ in pyridine.

$$[\alpha]_D^{20} = \frac{7.4810 \times -0.08°}{0.4180 \times 0.5} = -2.86°$$

$$[\alpha]_D^{20} = \frac{7.9450 \times -0.07°}{0.5028 \times 0.5} = -2.25°$$

$$[\alpha]_D^{20} = \frac{6.1186 \times -0.08°}{0.306 \times 0.5} = -3.2°$$

Since the first purification through the benzoyl derivative was so successful, it was thought that the repetition of the process would yield a cerasin with a still higher rotation. A product with $[\alpha]_D^{20} = -2.86°$ was therefore benzoylated and the methyl alcohol mother liquor saponified as above. The cerebroside, twice crystallized from methyl alcohol, showed a value for $[\alpha]_D^{20}$ of $-2.98°$.

$$[\alpha]_D^{20} = \frac{5.101 \times -0.10°}{0.3440 \times 0.5} = -2.98°$$

This indicates little or no purification. No better results were obtained with the acetyl derivative. The material before acetylation had a value of $[\alpha]_D$ of $-2.30°$, and the product after saponification of the acetyl derivative showed $[\alpha]_D = -2.34°$.

Hydrolysis of Cerasin Obtained by Fractionation.

The fraction most soluble in methyl alcohol was further fractionated out of pyridine and chloroform, which gave a product with a rotation of $-2.24°$.

$$[\alpha]_D^{20} = \frac{5.281 \times -0.17^\circ}{0.400 \times 1} = -2.24^\circ$$

30 gm. of this material were hydrolyzed with 450 cc. of 10 per cent hydrochloric acid and 75 cc. of 95 per cent alcohol. The acids obtained from this hydrolysis, worked up in the usual way, had the following composition:

0.1167 gm. substance gave 0.3293 gm. CO_2 and 0.1324 gm. H_2O.

	Calculated for $C_{24}H_{42}O_2$:	Calculated for $C_{22}H_{40}O_2$:	Found:
C	78.20	75.33	76.95
H	13.16	12.50	12.70

The fraction I a_2 (page 639) was extracted with dilute acetone (one part water to ten parts acetone). The insoluble part had a rotation of -3.45°.

$$[\alpha]_D^{20} = \frac{5.2834 \times -0.28^\circ}{0.400 \times 1} = -3.45^\circ$$

20 gm. of this material were hydrolyzed by boiling for 6 hours with 150 cc. of 98 per cent alcohol containing 7.5 cc. of sulfuric acid. On standing over night at room temperature, scales of ethyl lignocerate separated out.

0.1129 gm. substance gave 0.3240 gm. CO_2 and 0.1318 gm. H_2O.

	Calculated for $C_{26}H_{52}O_2$:	Found:
C	78.8	78.26
H	13.10	13.16

15 gm. of the same sample were then hydrolyzed with 150 cc. of 10 per cent hydrochloric acid and 15 cc. of 95 per cent alcohol. The fatty acids were obtained through the barium salts. The acids were then transferred into the ethyl ester by boiling in a solution of 100 cc. of 98 per cent alcohol containing 5 gm. sulfuric acid for 6 hours. On standing, scales separated out, with the following composition.

0.1150 gm. substance gave 0.3264 gm. CO_2 and 0.1306 gm. H_2O.

	Calculated for $C_{26}H_{52}O_2$:	Found:
C	78.80	77.40
H	13.10	12.71

Hydrolysis of Cerasin Obtained by the Benzoylation Process.

3 gm. of the material with $[\alpha]_D^{20} = -3.25°$ were heated in a sealed tube with 50 cc. of 3 per cent sulfuric acid at 100–105° for 18 hours. The acids were isolated through the barium salts and had the following composition.

0.1044 gm. substance gave 0.2940 gm. CO_2 and 0.1152 gm. H_2O.

	Calculated for $C_{26}H_{48}O_2$:	Calculated for $C_{25}H_{50}O_2$:	Found:
C	78.20	75.33	76.80
H	13.16	12.50	12.35

Acetyl Phrenosin.

Acetyl phrenosin was first prepared by Thierfelder.[6] 20 gm. of phrenosin, 20 gm. of fused sodium acetate, and 200 cc. of acetic anhydride were heated to gentle boiling for ½ hour. The excess of acetic anhydride was removed by distillation in vacuum and the semisolid residue dissolved in ether and water. The ether solution of the acetyl derivative was washed with water, with dilute alkali, and again with water, dried over sodium sulfate, and the ether concentrated on the water bath. The residue was taken up in hot dry methyl alcohol, in which it is very soluble, and from which it separates in a granular condition upon cooling. After the second crystallization from methyl alcohol, the yield was 14 gm. of a product with $[\alpha]_D = -10.4°$. The third crystallization gave a product with $[\alpha]_D = -11.07°$, which was not changed on further purification.

Acetyl phrenosin melts somewhat unsharply at 41–43°. Thierfelder gives the melting point as 39–41° and the rotation as −3°. Our product, dissolved in a mixture of equal parts of chloroform and methyl alcohol (by volume) showed the following rotation:

$$[\alpha]_D^{20} = \frac{7.0708 \times -0.39°}{0.4980 \times 0.5} = -11.07°$$

$$[\alpha]_D^{20} = \frac{6.5570 \times -0.43°}{0.5066 \times 0.5} = -11.08°$$

0.1052 gm. substance gave 0.2568 gm. CO_2 and 0.0892 gm. H_2O.

0.5000 gm. substance neutralized 4.35 cc. 0.1 N HCl.

0.500 gm. substance, used for an acetyl determination, required 28 cc. 0.1 N HCl, and in a second experiment, 28.2 cc.

0.673 gm. substance, in 22.56 gm. chloroform produced a rise in boiling point of 0.108°.

0.943 gm. substance, in 22.56 gm. chloroform, produced a rise in boiling point of 0.158°.

	Calculated for hexacetylphrenosin $C_{60}H_{106}NO_{18}$:	Found:	
C	66.68		66.57
H	9.80		9.49
N	1.29		1.22
Ac	23.8	24.08	24.24
Mol. wt.	1079	1003	960

One experiment was made to determine whether the same derivative could be prepared by the use of acetyl chloride. 20 gm. of phrenosin were dissolved in 150 cc. of pyridine, the solution was cooled to 0°C., 16 cc. of acetyl chloride were added in portions, and the mixture was allowed to stand 2 days at 0°C. The pyridine hydrochloride was filtered off, the solution washed with water, dilute hydrochloric acid, and then with water, dried with sodium sulfate, and concentrated. The residue was crystallized repeatedly from methyl alcohol. After the fourth crystallization, it had a rotation of −8.41°, and melted at about 40°.

$$[\alpha]_D^{20} = \frac{6.1218 \times -0.33°}{0.4794 \times 0.5} = -8.41°$$

The analysis indicates that it is probably a mixture of acetyl derivatives. It was not purified further.

0.1008 gm. substance gave 0.2484 gm. CO_2 and 0.0896 gm. H_2O.

	Calculated for Hexacetylphrenosin $C_{60}H_{106}NO_{18}$:	Triacetylphreno in $C_{54}H_{99}NO_{12}$:	Found:
C	66.68	67.94	67.22
H	9.80	10.46	9.96

Acetyl Cerasin.

20 gm. of a cerasin with $[\alpha]_D^{20} = -2.8°$, 20 gm. of sodium acetate, and 200 cc. of acetic anhydride were boiled under a reflux for ½

hour. The reaction product was worked up as described above, and the product then twice crystallized from methyl alcohol. This material did not show a change in rotation upon further purification from methyl alcohol. It is slightly more insoluble in methyl alcohol than is acetyl phrenosin, as stated by Thierfelder.[6] Acetyl cerasin melts at 54–56°, and in a mixture of equal parts of chloroform and methyl alcohol gave the rotation:

$$[\alpha]_D^{20} = \frac{5.9310 \times -0.60^\circ}{0.4324 \times 0.5} = -16.46^\circ$$

0.1035 gm. substance gave 0.2576 gm. CO_2 and 0.0932 gm. H_2O.
0.500 gm. substance neutralized 4.4 cc. 0.1 N HCl.

	Calculated for pentacetylcerasin $C_{67}H_{101}NO_{18}$:	Found:
C	67.87	67.75
H	10.10	10.00
N	1.40	1.23

As stated on page 639, the acetyl derivatives are not a suitable means of separating phrenosin and cerasin.

Benzoylphrenosin.

10 gm. of practically pure phrenosin were dissolved in 100 cc. of pyridine, cooled to room temperature, and treated with 12 cc. of benzoyl chloride. The reaction mixture was cooled under the water tap and then allowed to stand at 0°C. over night. The next day the pyridine hydrochloride was filtered off, the solution concentrated in vacuum at 50°C., and the resulting oily liquid poured, with stirring, into a large volume of about 2 per cent sodium hydroxide solution. The benzoylphrenosin separated as an oil on the sides and bottom of the dish. After washing with water and hydrochloric acid, the material was taken up in ether and dried with sodium sulfate. An alternative procedure is to dissolve the pyridine containing oily material in ether, then shake with dilute acid and alkali, then with water, and dry this solution. After removal of the ether the product is taken up in hot methyl alcohol. From a concentrated solution, the benzoyl derivative separates as an oil, which solidifies on cooling. From a sufficiently dilute solution it separates as colorless nearly crystal-

line material, which is easily filtered. It may also be obtained in a granular form from ethyl alcohol. It is easily soluble in acetone, benzene, petroleum ether, chloroform, acetic acid, and pyridine.

In an experiment in which 80 gm. of phrenosin were used, the benzoyl derivative was purified by crystallization from methyl alcohol, then from acetone (in which about 50 per cent of the material was lost), and then from methyl alcohol until the rotation was constant. The melting point as observed on this material was fairly sharp at 65–66°.

In methyl alcohol and chloroform the rotation is:

$$\text{I.}\quad [\alpha]_D^{20} = \frac{7.1484 \times +0.75^\circ}{0.5062 \times 0.5} = +21.1^\circ$$

$$\text{II.}\quad [\alpha]_D^{20} = \frac{6.1518 \times +0.92^\circ}{0.5320 \times 0.5} = +21.27^\circ$$

$$\text{III.}\quad [\alpha]_D^{20} = \frac{6.1060 \times +0.91^\circ}{0.5240 \times 0.5} = +21.20^\circ$$

Sample I was obtained from material which contained at least 75 per cent cerasin. Samples II and III were obtained from material considered nearly pure phrenosin. The analysis and molecular weight indicate that this material of constant rotation was probably a tribenzoylphrenosin.

0.1004 gm. substance gave 0.2704 gm. CO_2 and 0.0792 gm. H_2O.
0.1028 " " " 0.2784 " " " 0.0866 " "
0.1000 " " " 0.2707 " " " 0.0776 " "
0.5000 " " neutralized 4.40 cc. 0.1 N HCl.
0.5000 " " " 4.10 " 0.1 "
0.500 " " used for a benzoyl determination required 11.6 cc. 0.1 N NaOH, and in a second experiment 11.9 cc.
0.255 " " in 23.21 gm. chloroform raised the boiling point 0.026°.
0.682 " " as above, raised the boiling point 0.108°.
1.008 " " " " " " " " 0.160°.

	Calculated for tribenzoylphrenosin $C_{61}H_{58}NO_{12}$:	Found:		
C	72.65	73.45	73.89	73.81
H	9.28	8.83	9.43	8.69
N	1.23		1.23	1.12
C_6H_5CO	25.32		24.36	24.99
Mol. wt	1,139	1,037	1,066	1,092
Average			1,065	

An attempt was then made to prepare a hexabenzoyl derivative. 40 gm. of the above mentioned material were dissolved in 300 cc. of pyridine and treated with 400 cc. of benzoyl chloride. After working up as usual, the product was taken up in methyl alcohol. An oil formed which would not solidify even after standing several weeks. Since the material with constant rotation served our need the experiment was discarded.

Phrenosin from Benzoylphrenosin.

About 10 gm. of sodium were dissolved in 300 cc. of methyl alcohol and 20 gm. of benzoylphrenosin added with shaking (the benzoylphrenosin may also be added in acetone solution). A white precipitate gradually forms upon boiling (immediately, if an acetone solution is used). After boiling for 2 hours, the reaction product is cooled in the ice box, the precipitate filtered off, recrystallized from methyl alcohol, then from glacial acetic acid, and finally from a mixture of equal parts of methyl and ethyl alcohols.

The phrenosin crystallizes practically quantitatively for upon concentration of the mother liquor only sodium benzoate was obtained. The rotation of this material in pyridine corresponds well with that found for the phrenosin obtained by fractionation of the top fraction of the cerebroside mixture. Rosenheim gives +3.78° and +3.70°.

In pyridine the rotation was:

$$[\alpha]_D^{20} = \frac{+8.0000 \times 0.12^\circ}{0.5232 \times 0.5} = +3.67^\circ$$

Cinnamoylphrenosin.

10 gm. of phrenosin were dissolved in 100 cc. of pyridine and 15 gm. of cinnamoyl chloride added. After standing over night at 0°, the filtered solution was concentrated and worked up as described above. The resulting product was purified by crystallization from methyl or ethyl alcohol, or from acetone. Twice recrystallized from methyl alcohol, and once from acetone, the substance analyzed as a tricinnamoylphrenosin. Tricinnamoylphrenosin is slightly less soluble in organic solvents than the benzoyl derivative. It melts at 69–70°.

$$[\alpha]_D^{20} = \frac{7.2060 \times 0.75^\circ}{0.4976 \times 85} = +21.72^\circ$$

0.1028 gm. substance gave 0.2718 gm. CO_2 and 0.0868 gm. H_2O.
0.1016 " " " 0.2732 " " " 0.0870 " "
0.500 " " neutralized 4.3 cc. 0.1 N HCl.

	Calculated for tricinnamoylphrenosin $C_{75}H_{81}NO_{12}$:	Found:	
C	73.89	73.22	73.33
H	9.18	9.45	9.58
N	1.15	1.20	

p-Nitrobenzoylphrenosin.

20 gm. of phrenosin, dissolved in 200 cc. of pyridine, were treated with 32 gm. of *p*-nitrobenzoyl chloride, and the mixture was allowed to stand 24 hours at 0°. The reaction product was worked up as usual. The residue from the ether was extracted with hot acetone, the acetone removed on the steam bath, and the product extracted with boiling methyl alcohol. On cooling, the larger part of the nitrobenzoate separated as an oil. This was again extracted with boiling methyl alcohol, and the resulting cake twice crystallized from a large volume of methyl alcohol. *p*-Nitrobenzoylphrenosin is very soluble in acetone, and melts at 94–96°. Analysis indicates that tri-*p*-nitrobenzoylphrenosin is formed. The rotation in chloroform and methyl alcohol was:

$$[\alpha]_D^{20} = \frac{8.6510 \times 0.35^\circ}{0.4970 \times 0.50} = +12.18^\circ$$

0.1058 gm. substance gave 0.2542 gm. CO_2 and 0.0810 gm. H_2O.
0.1000 " " " 4.00 cc. N gas at 22°C. and 767 mm.

	Calculated for $C_{89}H_{102}N_4O_{24}$:	Found:
C	65.00	65.52
H	8.06	8.57
N	4.40	4.67

CEREBROSIDES.

V. CEREBROSIDES OF THE KIDNEY, LIVER, AND EGG YOLK.

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With the progress of our knowledge of the lipoids of the various organs of the animal body there comes a conviction that there does not exist much variation in the chemical composition of the individual lipoids with the variation of the organs or tissues from which they originate. Up to the present this has been found true in regard to cephalin[1] and sphingomyelin.[2] The present note contains data regarding the cerebrosides obtained from the same sources. The cerebrosides are found in organs other than the nervous system in very small proportions. Because of this the purification is associated with many difficulties. Furthermore, it is nearly impossible to obtain sufficiently large quantities of material for an exhaustive analysis.

For the present, one is satisfied to be able to show the presence of cerebrosides in several tissues—in the egg yolk, in the liver, and in the kidney—and to be able to state that the composition of these cerebrosides seems to be identical with that of the cerebrosides obtained from the nerve tissue. They contain the same sugar, galactose, the same base, sphingosine, and the same fatty acids, lignoceric and cerebronic.

It is not certain whether the two cerebrosides, phrenosin and cerasin, occur in the same proportions in nerve tissue and in the various other organs. The same remark applies regarding every other individual lipoid. It is possible that the proportions do vary with the variation of the organ.

Historically it must be mentioned that the first evidence of existence of cerebrosides in other organs than nervous tissue was

[1] Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1916, xxiv, 111.

[2] Levene, *J. Biol. Chem.*, 1916, xxiv, 69.

furnished by Hoppe-Seyler,[3] and later by Kossel and Freytag,[4] who discovered the presence of cerebrosides in pus cells. Recently Rosenheim and MacLean[5] showed, by the isolation of lignoceric acid and sphingosine from the so called carnaubon of Dunham,[6] that the kidney probably contained cerebrosides, but these were not isolated in a pure state.

The following table gives the composition and optical activity of the various cerebrosides thus far studied.

	Source.	C	H	N	$[\alpha]_D^{20}$
Phrenosin	Brain.	69.51	11.37	1.69	9.6* (3.6)**
Cerasin	"	69.53	11.70	1.68	−3.25**
Cerebrosides	Kidney.	69.60	11.30	1.68	+14.00*
"	Liver.	68.36	11.23	1.80	
"	Egg yolk.	69.32	11.19	1.89	+4.40*
Theory for phrenosin		69.65	11.23	1.70	
" " cerasin		71.20	11.33	1.73	

* In chloroform and methyl alcohol.

** In pyridine.

EXPERIMENTAL.

Kidney Cerebrosides.

The preparation of the cerebrosides from the kidney was accomplished by following the general plan outlined in the previous communication.[7] However, in place of pure cerebrosides which are obtained from nerve tissue by this process, the material prepared from the crude kidney "white matter" contained a considerable proportion of neutral fat, the removal of which was found very troublesome. A great aid for the purpose of purification of this material was found in the use of methyl ethyl ketone. By repeated crystallization from this reagent of the crude cerebrosides a substance was finally obtained which had all the properties

[3] Hoppe-Seyler, F., *Med.-Chem. Untersuch.*, 1866–71, 486.

[4] Kossel, A., and Freytag, F., *Z. physiol. Chem.*, 1893, xvii, 452.

[5] Rosenheim, O., and MacLean, H., *Biochem. J.*, 1915, ix, 103.

[6] Dunham, E. K., *J. Biol. Chem.*, 1908, iv, 297. Dunham, E. K., and Jacobson, C. A., *Z. physiol. Chem.*, 1910, lxiv, 302.

[7] Levene and West, *J. Biol. Chem.*, 1917, xxxi, 635.

of the mixed cerebrosides. This material gave with orcin the typical test for galactose, and on hydrolysis yielded sphingosine and the typical fatty acid mixture.

0.0984 gm. substance gave 0.2456 gm. CO_2 and 0.0972 gm. H_2O.
0.500 " " neutralized 6 cc. 0.1 N HCl.

	Calculated for phrenosin:	Found:
C	69.65	69.60
H	11.24	11.30
N	1.70	1.68

The optical rotation of the substance, in a mixture of equal parts of chloroform and methyl alcohol (by volume) was:

$$[\alpha]_D^{20} = \frac{9.5312 \times 0.26^\circ}{0.3550 \times 0.5} = +14.00^\circ$$

Hydrolysis of the Mixed Cerebrosides.

1.5 gm. of the mixed cerebrosides were heated with 75 cc. of 3 per cent sulfuric acid for 24 hours in a sealed tube at 105°. The base and acids were separated and prepared for analysis as described in a previous article.

The acids had the following composition:

0.1012 gm. substance gave 0.2978 gm. CO_2 and 0.1098 gm. H_2O.

	Calculated for $C_{25}H_{50}O_2$:	Calculated for $C_{24}H_{48}O_2$:	Found:
C	75.33	78.20	75.40
H	12.50	13.20	12.18

Thus the acid was apparently nearly pure cerebronic acid, $C_{25}H_{50}O_2$.

The base was transformed into the sulfate and gave the following figures on analysis.

0.0990 gm. of substance gave 0.2264 gm. CO_2 and 0.0910 gm. H_2O.

	Calculated for $(C_{17}H_{37}NO_2)_2H_2SO_4$:	Found:
C	61.08	62.36
H	10.78	10.29

Crude sphingosine sulfate, previous to crystallization, very frequently gives analytical data as in the present experiment. There is little doubt that the base of the kidney cerebrosides is sphingosine.

Liver Cerebrosides.

Desiccated and pulverized liver tissue was allowed to stand over night with 95 per cent alcohol and then filtered. The residue was repeatedly extracted with boiling alcohol, each extraction lasting about ½ hour. The combined extracts, on standing in the refrigerator, at 0°, gave a dark, nearly black deposit, which corresponds to the "white matter" of the brain extracts. This deposit was extracted in the cold progressively with acetone, alcohol, and ether. The still dark but quite dry mass was fractionated into two parts by dissolving it in hot pyridine and allowing it to cool to room temperature. The mother liquor containing the cerebrosides was concentrated and poured into acetone. The precipitate thus obtained was still very dark. For further purification it was boiled with hot alcohol; a small part remained insoluble. The solution was decanted and a concentrated solution of barium hydroxide was added as long as a precipitate formed. This mixture was allowed to stand in the ice box, and the precipitate which formed was repeatedly extracted with boiling alcohol. The extracts, upon cooling to 0°, gave a precipitate which had the appearance and properties of the brain cerebrosides. This product was then repeatedly extracted with ether, when analysis showed that it was still contaminated with large amounts of neutral fat. The purification was then continued by extraction with ether and by crystallization from acetic acid. The product, however, persisted in containing neutral fat. Finally, the product was dissolved in hot methyl ethyl ketone, from which it settled out on cooling. This was repeated three times, the product then having the composition of cerebrin. Because of the great losses connected with the purification it was not possible to obtain sufficient material for hydrolysis. The test for galactose was positive and there is little doubt that we are dealing with a characteristic cerebroside mixture.

0.1006 gm. substance gave 0.2522 gm. CO_2 and 0.1010 gm. H_2O.

	Calculated for phrenosin:	Found:
C	69.65	68.36
H	11.24	11.23

Egg Cerebrosides.

Egg yolk (dried commercial egg yolk was used in all the work) was thoroughly extracted with acetone at room temperature, to remove egg oil. The material was then extracted with boiling alcohol, repeatedly, as in the former preparations. The combined alcoholic extracts were concentrated to a small volume, and repeatedly treated with acetone, to complete the removal of the egg oil. The acetone-insoluble fraction (lecithin, cephalin, cerebrosides, and saturated phosphatides) was extracted with ether. A small part did not go into solution. The ether suspension was centrifuged, the insoluble material suspended in acetone, filtered off, and again extracted with ether. The insoluble material then corresponded to "white matter" previously mentioned, and is the material analyzed by Stern and Thierfelder[8] and considered impure diaminomonophosphatide. It was later given the name *albin* by Bing and Ellermann.[9]

This was fractionated out of pyridine as described above. The cerebroside fraction was crystallized out of glacial acetic acid and the neutral fat removed by repeatedly extracting with acetone at 50°, and finally by crystallization from methyl ethyl ketone. This material possessed all the physical properties of the mixed cerebrosides, and gave the galactose test with orcin.

0.1020 gm. substance gave 0.2476 gm. CO_2 and 0.0974 gm. H_2O.
0.5000 " " neutralized 6.75 cc. 0.1 N HCl.

	Calculated for phrenosin:	Found:
C	69.65	69.32
H	11.24	11.19
N	1.70	1.89

The optical activity of the material in a mixture of methyl alcohol and chloroform was:

$$[\alpha]_D^{20} = \frac{12.4370 \times 0.11^\circ}{0.3062 \times 0.5} = +4.40^\circ$$

[8] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.
[9] Bing, H. J., and Ellermann, V., *Biochem. Z.*, 1912, xlii, 289.

Hydrolysis of Egg Cerebrosides.

1.5 gm. of the substance were heated in a sealed tube with 75 cc. of 3 per cent sulfuric acid for 24 hours at 105°.

The acids and bases were prepared in the manner described above.

0.1000 gm. substance gave 0.2708 gm. CO_2 and 0.1070 gm. H_2O.

	Calculated for $C_{28}H_{50}O_8$:	Found:
C	75.33	74.52
H	12.50	12.09

The base was analyzed as the sulfate.

0.0905 gm. substance gave 0.2024 gm. CO_2 and 0.0810 gm. H_2O.

	Calculated for $(C_{17}H_{35}NO_2)_2H_2SO_4$:	Found:
C	61.08	60.99
H	10.78	10.01

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